



MICROBES AND TOLL-LIKE RECEPTORS IN ORAL LICHENOID DISEASE AND ORAL SQUAMOUS CELL CARCINOMA

Peter Rusanen

Academic dissertation

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Department of Bacteriology and Immunology
Haartman Institute
Faculty of Medicine
University of Helsinki
Helsinki, Finland

SUPERVISED BY:**Riina Richardson**

DDS, PhD, FRCPath, FECMM, PGCertMedEd

Division of Infection, Immunity and Respiratory Medicine, Faculty of Biology, Medicine and Health, University of Manchester; AND Department of Infectious Diseases, Manchester University Foundation Trust, Wythenshawe Hospital, UK

Emilia Marttila

MD, DDS, PhD

Department of Oral and Maxillofacial Diseases, Helsinki University Hospital and University of Helsinki, Finland

REVIEWED BY:**Sohvi Hörkkö**

MD, PhD, eMBA, Professor in Immunology

Unit of Biomedicine, University of Oulu, Finland

Joonas H. Kauppila

MD, PhD, Docent

Upper Gastrointestinal Research, Department of Molecular Medicine and Surgery, Karolinska Institutet, Sweden and Unit of Surgery, Anesthesia and Intensive Care, University of Oulu

OPPONENT:**Justus Reunanen**

PhD, Docent

Biocenter Oulu; AND Cancer and Translational Medicine Research Unit, University of Oulu, Finland

CUSTOS:**Tuula Salo**

DDS, PhD, Professor of Oral Pathology

Department of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Hospital, Helsinki; AND Cancer and Translational Medicine Research Unit, University of Oulu, and Medical Research Centre Oulu University Hospital, Oulu, Finland

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1 ORIGINAL PUBLICATIONS

I. Rautemaa R, Rusanen P, Richardson M, Meurman JH. Optimal sampling site for mucosal candidosis in oral cancer patients is the labial sulcus. *J Med Microbiol.* 2006 Oct;55(Pt 10):1447-51.

II. Rusanen P, Siikala E, Uttamo J, Richardson M, Rautemaa R. A novel method for sampling the microbiota from the oral mucosa. *Clin Oral Investig.* 2009 Jun;13(2):243-6.

III. Marttila E, Uttamo J, Rusanen P, Lindqvist C, Salaspuro M, Rautemaa R. Acetaldehyde production and microbial colonization in oral squamous cell carcinoma and oral lichenoid disease. *Oral Surg Oral Med Oral Pathol Oral Radiol.* 2013 Jul;116(1):61-8.

IV. Rusanen P, Marttila E, Uttamo J, Hagstrom J, Salo T, Rautemaa-Richardson R. TLR1-10, NF-kappaB and p53 expression is increased in oral lichenoid disease. *PLoS One.* 2017 Jul 17;12(7):e0181361.

Publication III has been used as a part of dissertation by Dr J. Uttamo.

2 ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase enzyme
BA	Lysed blood agar
CFU	Colony forming unit
CLED	Cysteine-, lactose-and electrolyte-deficient agar
DAMP	damage-associated molecular pattern
DNA	Deoxyribonucleic acid
EBV	Ebstein-Barr virus
EPS	extracellular polymeric substances
FAA	Fastidious anaerobe agar
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HSP	heat shock protein
IL	Interleukin
LAM	lipoarabinomannan
LPS	Lipopolysaccharides
mRNA	Messenger ribonucleic acid
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MyD88	adaptor protein in Toll/IL-1 receptor family signalling
NF- κ B	Nuclear factor- κ B
NV	Neomycin-vancomycin blood agar
OLD	Oral lichenoid disease
OLL	Oral lichenoid lesion
OLP	Oral lichen planus
OSCC	Oral squamous cell carcinoma

PAMP	pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PCR	Polymerase chain reaction
PRRs	Pattern recognition molecules
RNA	Ribonucleic acid
rs	Spearman's Rho
SEM	Standard error of mean
SP	Saboraud dextrose agar
TIR	TLR/IL-1 receptor
TIRAP	TIR domain-containing adaptor protein
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter protein inducing IFN- β
TLR	Toll-like receptor
WHO	World Health Organization

3 ABSTRACT

Oral lichenoid disease (OLD) encompasses oral lichen planus (OLP) and oral lichenoid lesion (OLL), which are chronic T-cell-mediated mucocutaneous inflammatory disorders of unknown aetiology. Both OLP and OLL are classified as potentially malignant disorders. Although various antigens have been considered, it is not known what triggers the inflammatory response of T-cells. Suggested predisposing factors include stress, genetic factors, trauma, viral, fungal and bacterial infection.

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the oral cavity. It is a multifactorial disease with no single clearly recognizable cause. Chronic inflammation is one of the most important causes of OSCC. Chronic oral candidiasis has also been associated with oral carcinoma in several studies. It is still debatable whether microbial infections initiate cancer or is the preexisting cancer colonized by microbes secondarily.

Acetaldehyde is the first metabolite of ethanol and it is carcinogenic. Acetaldehyde is also produced by microbes and poor oral hygiene increases acetaldehyde production. Recent studies of the oral microbial acetaldehyde production are mainly based on uncultured saliva samples. Saliva and mouth rinse samples are often used for general sampling but do not represent the microbes at a specific lesion or site.

Toll-like receptors (TLRs) and nuclear factor- κ B (NF- κ B) signalling transduction pathway play important roles in the pathogenesis of several chronic inflammatory diseases. Tumour suppressor protein p53 regulates TLR expression.

It was not known clearly what the optimal sampling site and method to study the microbial colonisation on mucosal lesions is and what impact specific microbial colonisation has on TLR expression. In addition, the immunohistochemical localisation of all TLRs in OLD was not established. Therefore, the aim of the first study was to investigate how the method and site of microbial sampling affect the discovery of *Candida* species on OSCC lesions. The objective of the second study was to develop a site-specific sampling method that would give quantitative results for samples from the oral mucosa. The aim of the third study was to explore lesion specific microbes and their ability to produce acetaldehyde in OSCC and OLD patients. Furthermore, the aim of the fourth study was to investigate the

immunohistochemical staining and tissue localization of TLR1-10, p53 and NF- κ B in mucosal biopsies from patients with OLD.

In the first study, four different sampling methods in oral cancer patients were compared for culture of yeasts. In the second study, two site-specific sampling methods, filter paper and swab, were compared for microbiological analyses of the healthy oral mucosa. The filter paper sampling method was developed for the second study. In the third study, microbial samples from OSCC and OLD patients for microbiological analyses and acetaldehyde measurement were obtained using the filter paper sampling method. In the fourth study, oral mucosal biopsies from patients with OLD and from healthy controls were analysed for the expression of TLR1-10, NF- κ B and p53 by immunohistochemistry.

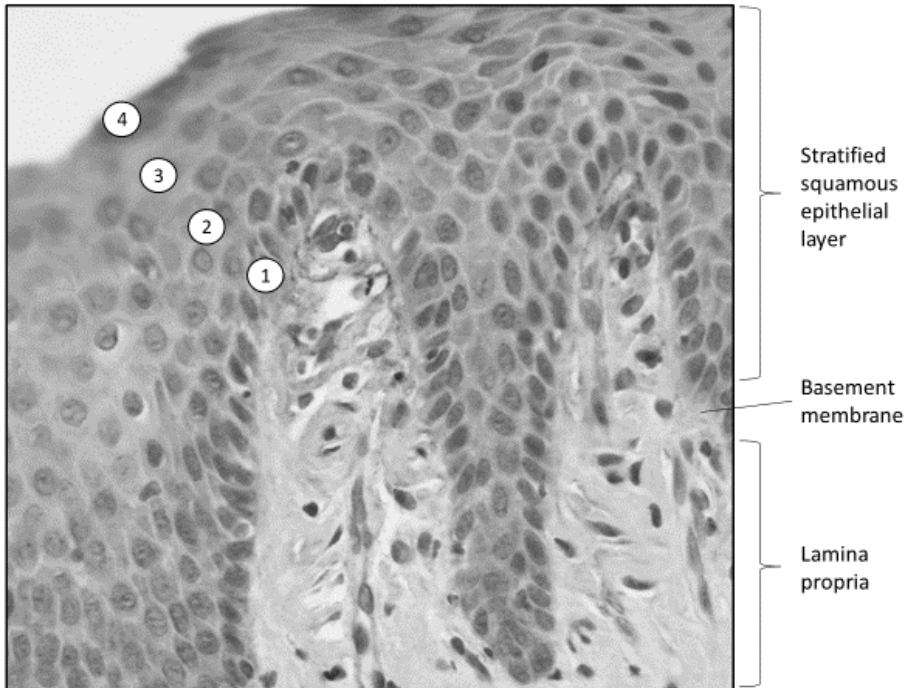
This work has demonstrated that after cancer treatment, the incidence of *Candida albicans* was found to be increased and a shift from *C. albicans* to other *Candida* species was found. The optimal sampling site for *Candida* in these patients was found to be the labial sulcus. Moreover, the filter paper sampling method was found to be an ideal technique for obtaining quantitative data from defined areas of the oral mucosa. Based on the filter paper sampling method, it was detected that the bacterial composition on OSCC and OLD lesions differed from that of the healthy appearing contralateral mucosa and from healthy controls. *Candida* colonization was higher in OSCC and OLD lesions and patients with *Candida* colonization produced significantly more frequently mutagenic amounts of acetaldehyde. The staining intensity of several TLRs was markedly stronger throughout the epithelium and in the basement membrane zone of OLD samples. Likewise, the staining for NF- κ B and p53 were more intense in OLD samples compared to the control samples. We did not find any correlations between the microbial samples and the immunostaining of TLRs.

In conclusion, this study showed that the composition of lesional microbes differs on OSCC and OLD lesions compared to the healthy appearing mucosa and to the healthy controls. Furthermore, the composition rather than the number of microbes is a significant factor that influences the production of carcinogenic level of acetaldehyde. Our results indicate that acetaldehyde and *Candida* colonisation may have an impact on TLR4 expression that may play a role in OSCC pathogenesis. The role of soluble TLR forms in the basement membrane zone calls for further studies.

4 REVIEW OF THE LITERATURE

4.1 ORAL MUCOSA

Fig. 1. Oral mucosa consists of (1-4) epithelium and lamina propria. The layers in the epithelium are (1) stratum basale, (2) stratum spinosum, (3) stratum granulosum and (4) stratum corneum. Modified from Rusanen *et al.* 2017 (1).



The oral mucosa is the inner side of the oral cavity and consists of stratified squamous epithelium and an underlying connective tissue termed lamina propria. The flattened keratinocytes in stratified squamous epithelium are arranged in layers maintaining a structural integrity and separate the body from its environment. The different layers of the epithelium are shown in figure 1. The basal cells form a proliferating layer that is attached to the basement membrane through hemidesmosomes. The keratinocytes of the basal cells differentiate into stratum spinosum and migrate superficially. In some region of the mouth the keratinocytes in the stratum granulosum differentiate into nonvital keratinized cells forming the stratum corneum. Keratinized stratified squamous epithelium can be found in the hard palate, dorsum of the tongue and attached gingiva and non-keratinized epithelium can be found elsewhere in the oral cavity; including buccal, labial, and alveolar mucosa as well as the floor of the mouth. The basement membrane under the basal cells is a thin fibrous extracellular matrix and it

anchors the epithelium into the connective tissue underneath. The connective tissue is a loose cell rich layer that consists of a network of collagen and elastic fibres which are produced by the fibroblasts. It is also rich in many cells specific to immune, inflammatory, and vascular system, lymphatic and blood vessels, and nerves. (2)

Oral mucosa forms a mechanical barrier against microbes and it serves as a first defence against infection. In addition, constant desquamation of oral epithelium also helps remove bacteria and other infectious agents that have adhered to the epithelial surfaces. Together with epidermal and circulating immune cells keratinocytes participate in the regulation of inflammatory reaction and immune responses. (2)

4.2 HUMAN ORAL MICROBIOME

The oral cavity is colonized by a set of microorganisms, including bacteria, archaea, fungi, and viruses and the composition of these microorganism varies according to the unique retention site of the oral cavity (3, 4). Approximately 280 bacterial species have been isolated from the oral cavity in culture and the use of culture-independent molecular methods have identified over 700 species (5). The development of molecular sequencing techniques has provided extensive information of the microbial diversity in composition and genome content (3, 4).

Biofilms are highly organised microbial communities embedded in a self-produced matrix of extracellular polymeric substances (EPS) that adhere microbes to each other and to the surfaces and provide shelter and accumulation of nutrients to the microbes (6). Through the intercellular physical and social interaction together with the EPS the biofilm is distinct to free living microbes and is not predictable from the study of free-living bacterial cells (6). The biofilm enables an enhanced resistance or tolerance to antibiotics and other antimicrobial agents compared with free-living bacterial cells (6). The composition of biofilms is affected by many factors like age, diet, oral hygiene, host immune responses, and medication and it varies with the balance between health and disease conditions (7).

4.2.1 Bacteria

Various analysis have revealed that 96% of oral bacteria in a healthy oral cavity constitutes of six major phyla: Actinobacteria, Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes and Spirochaetes (4, 8, 9). The other major

constituents of the core microbiome of the oral cavity include *Actinomyces*, *Atopobium*, *Corynebacterium*, *Rothia* of Actinobacteria; *Bergeyella*, *Capnocytophaga*, *Prevotella* of Bacteroidetes; *Granulicatella*, *Streptococcus*, and *Veillonella* of Firmicutes; *Campylobacter*, *Cardiobacterium*, *Haemophilus*, *Neisseria* of Proteobacteria; Saccharibacteria, and Fusobacteria (9). The oral microbial composition differs in between healthy individuals and in between different niches in oral cavity (7). The dominant groups of bacteria in supragingival dental plaque are Firmicutes and Actinobacteria while anaerobic *Prevotella* and *Capnocytophaga* of Bacteroidetes are found in the niches of dorsal and lateral surfaces of tongue (4). Microbes attached to surfaces continuously shed into the saliva and each millilitre of saliva contains an average 1.4×10^8 colony forming units (CFU) bacteria from which the *Streptococcus* among Firmicutes is the most abundant bacteria (9). The composition of bacteria in saliva varies extensively and patients with dental periodontitis (10), caries (11) and oral squamous cell carcinoma (12) show a different salivary bacterial composition and distribution from healthy populations (7). With the development of periodontal disease, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola* are the most abundant bacteria (13). *Streptococcus mutans* has been regarded as a specific pathogen in dental caries but also *Streptococcus*, *Lactobacillus*, *Actinomyces*, *Propionibacterium*, and *Veillonella* were also detected at a higher amount in caries-active adults (14). While the microbial composition varies in between different niches and sites in oral cavity, it underlines the importance of choosing a correct sampling strategy which strictly complies with the aim of the microbiological trial (15).

4.2.2 Fungi

Fungi comprise a minor component of the oral microbiome and for most people, yeasts are a part of the normal oral flora (16). *Candida albicans* is the most frequently detected fungal species in the oral cavity and other less common species include *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. dubliniensis*, *C. stellanoidea* and *C. kefyr* (17). In healthy individuals, the amount of fungi is controlled by specific and non-specific defence mechanisms of the saliva and the oral mucosa, as well as by competition among oral microbes (18). However, if the balance of the normal flora is disrupted or the local or systemic immune defences mechanism are compromised, *Candida* often become pathogenic, causing mucosal disease (19). *Candida* is also involved in other diseases like caries and it is highly associated with the severity of chronic periodontitis (7). Human infections caused

by *Candida* range from the more common oral thrush to fatal, systemic superinfections in patients who are afflicted with other diseases (20). Chronic oral candidiasis has been associated with oral carcinoma in several studies (21-23). Common predisposing factors that cause candidiasis are reduced saliva secretion due to medication or radiotherapy, primary or secondary deficiencies of humoral or cell mediated immunity, local mucosal diseases, and the use of wide-spectrum antibiotics (19). Although all *Candida* species cause a similar mucosal infection, there are remarkable differences in the antifungal susceptibilities and invasiveness among species (24, 25).

4.2.3 Viruses

Viruses are small infectious agents that require living cells of other organisms like in the cells of animals, plants, bacteria, and fungi for replication. All viruses contain the following two components: a nucleic acid genome and a protein capsid that covers the genome. Together this is called the nucleocapsid. In addition, many animal viruses contain a lipid envelope. The entire intact virus is called the virion. Viruses do not have a cellular structure or their own metabolism and therefore cannot reproduce outside a host cell (26). The oral virome contains a range of viruses and their presence may be closely related to oral microbial diversity (27). Viruses that have infected bacteria may have a substantial capacity to alter human bacterial communities and may have a role in both health and in disease, such as chronic periodontitis (27, 28).

Viruses have extensive effects on the host cell. Most viral infections eventually cause death to the host cell through different mechanisms, such as cell lysis, alterations to the cell's surface membrane, or apoptosis. Some viruses can stay latent and inactive causing no apparent changes to the infected cell. Oral viruses are associated to diseases, such as herpes zoster (varicella zoster virus), herpetic gingiva-stomatitis and herpes labialis (herpes simplex virus), and papillomas (human papilloma virus) (29, 30). In addition, Epstein-Barr (EBV) virus can cause oral ulcers, multiple palatal petechia or infrequently gingival ulcerations (31). In active periodontal lesions different viruses can be detected, such as human cytomegalovirus (HCMV), EBV type 1-2, herpes simplex virus (HSV) type 1, and human herpes virus types 6-8 (7, 32).

4.2.4 Energy metabolism of the oral microbes

Bacteria, fungi and parasites uptake and utilize inorganic or organic compounds required for growth and maintenance of cellular steady state. To maintain their

basic functions and to replicate when in an appropriate milieu, these microbes must generate energy through substrate oxidation and dissimilation reactions. These reactions are catalysed within the bacterial cell by integrated enzyme systems. Chemical energy generated by substrate oxidations is conserved by formation of high-energy compounds such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP) or compounds containing the thioester bond such as acetyl-CoA or succinyl-CoA (33). Bacteria, like mammalian and plant cells, use ATP or the high-energy phosphate bond as the primary chemical energy source to synthesize the new complex organic compounds needed by the cell. Bacteria require also B-complex and vitamins as functional coenzymes for many oxidation-reduction reactions. For the ATP synthesis the most oxidized compounds are carbohydrates (particularly glucose), protein and lipids. (33)

In bacteria, glycolysis can occur in one of several pathways by which bacteria dissimilate glucose. The complete oxidation of glucose may involve three fundamental biochemical pathways: glycolytic pathway, citric acid cycle or membrane-bound electron transport oxidations coupled to oxidative phosphorylation. Citric acid cycle is a series of chemical reactions used by all aerobic microbes. The glycolytic pathway is most commonly associated with anaerobic or fermentative metabolism in bacteria and yeasts. In aerobic respiration the molecular O_2 serve as terminal acceptor of electrons and in anaerobic respiration, NO_3^- , SO_4^{2-} , CO_2 , or fumarate can serve as terminal electron acceptors. The result of the respiratory process is the complete oxidation of carbohydrate into CO_2 and H_2O . (33, 34)

In fermentation, energy is generated in anaerobic condition through the dehydrogenation reactions that occur as glucose is broken down enzymatically. For most microbial fermentations, glucose dissimilation occurs through the glycolytic pathway and the organic compound most commonly generated is pyruvate or a compound derived enzymatically from pyruvate, such as acetaldehyde and acetyl-CoA. Acetaldehyde can then be reduced by nicotinamide adenine dinucleotide ($NADH + H^+$) to ethanol, which is excreted by the cell. (33, 34)

4.2.5 Acetaldehyde

Acetaldehyde is the first metabolite of ethanol and this reaction is catalysed by alcohol dehydrogenases (ADH). Many bacteria possess marked ADH activity and alcohol-derived acetaldehyde exposure may occur in the oral cavity

independently from liver metabolism (35). Several studies performed in humans found higher levels of acetaldehyde in saliva compared to those found in blood after alcohol consumption (36-39). Acetaldehyde is reactive and toxic and has been classified by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen (40, 41). The carcinogenic concentration of aldehyde as low as 100µM can be measured in saliva after moderate alcohol consumption (39-42). Acetaldehyde interferes with DNA synthesis and repair at many sites and these alterations may result in tumour development (36, 38, 39). Acetaldehyde also induces inflammation and metaplasia of the tracheal epithelium and enhances cell injury (38). While cellular ADHs represent an important source of acetaldehyde, it can also be formed in the human oral cavity by the action of microorganisms such as oral *Streptococci*, *Neisseria* spp. and *Candida* (42-46). Thus, poor oral hygiene increases acetaldehyde production into saliva (43). Acetaldehyde can also be found in tobacco smoke. For oral squamous cell carcinoma (OSCC), smoking and poor oral hygiene are risk factors that amplify the malignant effects of simultaneous alcohol consumption (42, 47, 48).

Recent studies of the oral microbial acetaldehyde production are mainly based on uncultured saliva samples (36, 42, 43, 45). Saliva and mouth rinse samples are often used for general sampling but do not represent the acetaldehyde producing microbes at a specific lesion or site.

4.2.6 Sampling and culture of oral microbes

The oral cavity includes several distinct sites, such as teeth, tongue, lip, cheek, gingival sulcus, attached gingiva, hard palate, and soft palate which are colonized by distinct microbes (5). In health, there is a balance between oral microorganisms and the local defensive mechanisms and changes that alter that balance may lead to disease. Factors that may alter this balance include changes in the integrity of the epithelium, changes in secretion of saliva or in the immune system (5). Oral microorganisms may be the primary cause of oral lesions or secondary invaders in an already established mucosal lesion (49).

The oral microflora has been shown to differ both in spectrum and quantity in healthy mucosa compared to, for example, oral cancer lesions (44, 47, 50, 51), aphthous ulcers (52), and chronic mucosal oral diseases (49). Samples for microbiological analysis should be collected from a site representative of the active disease process (8). A sterile swab is the most commonly used method for sampling a mucosal lesion (5). However, although swab samples can detect

microbes on a certain area, it is still a quantitative estimate and the technique is difficult to standardize. Mouth rinse and saliva samples are often used for general sampling, but these methods cannot identify the site of infection and may miss adherent species (53). In addition, mouth rinse and saliva samples may not be useful for patients lacking tongue or lip function or for patients with lowered saliva secretion, for example, due to the radical changes during treatment for oral cancer (51, 53).

For microbial culture, the sample is plated onto non-selective and selective media. The non-selective media support the growth of many oral species and the selective media is used to help to identify specific species. The culture plates are incubated under appropriate atmospheric conditions up to 7 days after which different colonies can be detected and analysed. Microbes are identified using Gram staining, microscopy, and biochemical tests (54). In recent years matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry has been demonstrated as a fast and cost-effective identification method in clinical microbiology laboratories (55-57). The identification of cultured bacteria or yeasts by MALDI-TOF mass spectrometry is based on ionization technique where the mass spectrum of an ionizing molecule is measured by a detector.

4.3 ORAL MUCOSAL IMMUNE RESPONSES

The oral cavity is constantly protected from invading microbes, foreign antigens, and toxic agents by non-specific and specific immune mechanisms. Oral mucosa serves as a barrier against the invading microbes and several defence mechanisms of the saliva, as well as the competition among oral microbes, constantly reduces the number of oral microbes. In health, the immune system recognizes and removes the invading pathogens and can distinguish the body's own cells from invading pathogens and infected cells. (5)

The human immune system can be divided into innate and adaptive immunity. In the adaptive immunity the T and B lymphocytes play a major role. B lymphocyte activation begins when it binds to an antigen after which it differentiates into an antibody secreting plasma cell. T lymphocytes are mobilized when they encounter an antigen presenting cell (APC) such as dendritic cell, B lymphocyte or macrophages. Other cell types such as keratinocytes can also present an antigen. Depending on the T lymphocyte subgroup activated they can regulate immune responses, or they can directly attack infected or cancerous cells carrying foreign

peptides on their surfaces. T lymphocyte subgroups include helper, killer, regulatory, and potentially other T-cell types, such as auto-cytotoxic CD8+ T-cells. The adaptive immune system develops slowly but it is highly specific to a certain pathogen and it can also provide long-lasting protection. In contrast, the innate immune system does not have an immunological memory but once activated by foreign invaders the innate immune system provides an immediate response. The innate immune system includes e.g. cytokines, serum complement and broadly distributed phagocytic cells and leucocytes that recognise pathogen through pattern recognition receptors (PPRs). (58, 59)

If a pathogen invades the oral mucosa, an inflammatory reaction develops, and the immune system aims to destroy the invader. Some microbes can evade the immune system and cause a chronic infection despite the concerted activity of the immune mechanism. In fact, many inflammatory conditions and immunological disorders have been linked to a specific microorganism (60, 61). The role of chronic inflammation and the innate immune system in the development of cancer is widely recognized and a strong link between chronic inflammation and many types of cancers have been reported (62).

4.3.1 Toll-like receptors

Toll-like receptors (TLRs) are receptors of the innate immunity. They are expressed on various immune cells, such as macrophages and dendritic cells but are also present in non-immune cells, such as keratinocytes of the skin and oral mucosa (63). TLRs belong to the pattern recognition receptors (PPRs) which recognize molecules of pathogens known as pathogen-associated molecular patterns (PAMPs) and trigger the release of inflammatory cytokines and type I interferons for host defence (63). The responses of the TLRs are important not only to eliminate pathogens but also to develop the pathogen-specific adaptive immunity, which is mediated by B- and T-cells (64). TLRs also maintain tissue homeostasis by regulating the inflammatory and tissue repair responses to injury (65).

TLRs regulate a wide range of biological responses including inflammatory and immune responses during carcinogenesis (66). TLRs may promote carcinogenesis through proinflammatory, anti-apoptotic, proliferative and profibrogenic signals in either the tumour microenvironment or tumour cells themselves (66). One important tumour-promoting signalling pathway induced by TLR signalling is the transcription factor NF- κ B, as described below. Association of TLRs with the risk

of oral squamous cell carcinoma (OSCC) is still conflicting and the available evidence is weak due to the sparseness of data or disagreements among the reported investigations (67). However, high expression of TLR4 was significantly associated with the outcome of patients with solid cancers (68).

4.3.1.1 Structure and localization of TLRs

TLRs are transmembrane receptors with a leucine rich extracellular domain that are involved in the ligand recognition. The intracellular domain is known as TLR/IL-1 receptor (TIR) domain which is analogous to that of interleukin receptors and is essential for signal transduction (69). In human, so far, ten TLRs have been identified of which TLR1,2,4,5,6 are expressed on the cell surface and TLR3,7,8,9 are expressed on intracellular vesicles, such as the endosome (63). The cellular localization of TLR10 has not yet been well characterized but according to Lee *et al* 2018, TLR10 could be detected on the cell surface but was more abundant intracellularly (70). The cellular localization of TLRs correlates with their functions in sensing invading pathogens (70). Soluble forms of several TLRs have been detected in body fluids, such as breast milk, plasma and saliva (71-74). TLRs recognize various PAMPs derived from viruses, bacteria and fungi and protozoa (63). The ligand diversity is further broadened by forming heterodimers, such as TLR1/2 or TLR2/6 and TLR4/MD-2 (63, 75). TLR1-10 localization, ligands and signalling are presented in figure 2.

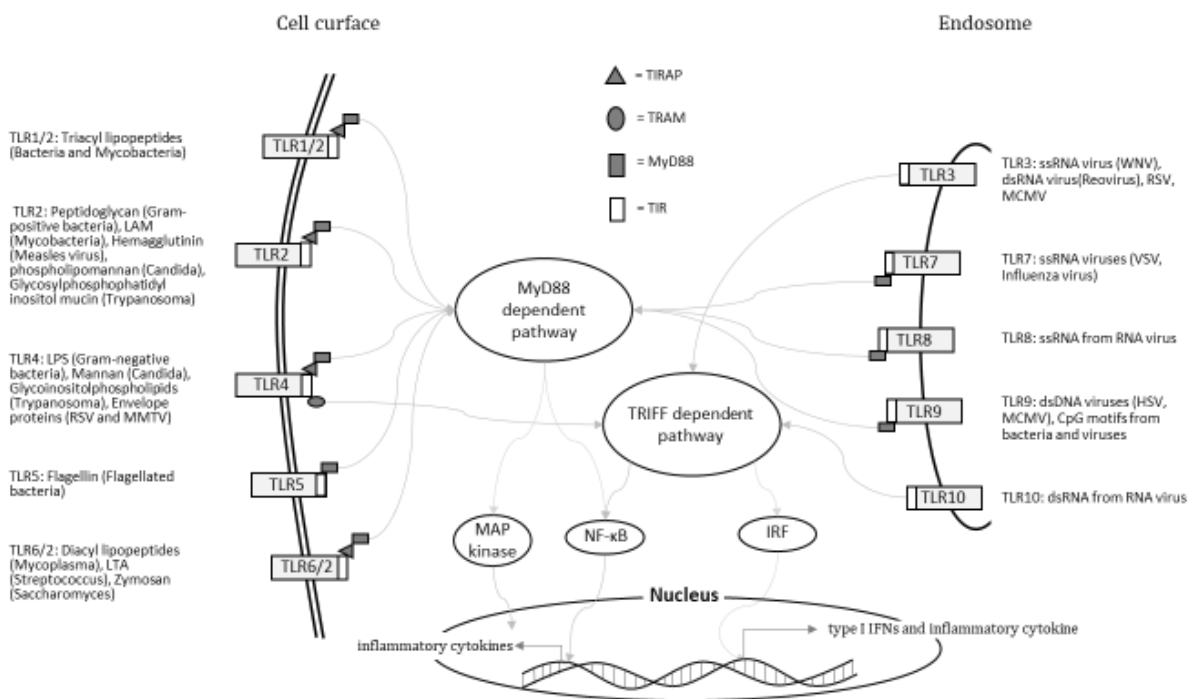
4.3.1.2 TLR ligands

Representative PAMPs of bacterial cell wall components are recognized by different TLRs: Lipopolysaccharides (LPS) of gram-negative bacteria are recognized by TLR4; peptidoglycans and several lipoproteins from gram-positive and gram-negative bacteria or lipoarabinomannan (LAM) from mycobacteria are recognized by TLR2; diacyl or triacyl lipopeptides from bacteria, mycobacteria, and mycoplasma are recognized by TLR1/2 or TLR2/6 (76). Although, Mycoplasma does not possess cell walls, its plasma membrane also contains several lipopeptides which are recognized by TLR2, TLR2/1 or TLR2/6 (76). TLRs can also recognize proteins, such as flagellin from flagellated bacteria (TLR5). Viruses are important PAMPs which contain envelope proteins and nucleic acids (single stranded (ss) or double stranded (ds) RNA or ss/ds DNA) and are recognized by various TLRs. Envelope proteins from viruses are recognized by TLR2, TLR4, and TLR6 and virus derived nucleic acids are recognized by TLR3 (dsRNA), TLR7 and TLR8 (ssRNA) and TLR9 (DNA)(77, 78). Several components

of *Candida* spp. such as β -glucan, chitin, mannan, proteins, and nucleic acids are recognized by at least five TLRs (TLR2, TLR4, TLR6, TLR7, and TLR9)(77). The cell surface located TLR2 and TLR4 play crucial roles in the recognition of the *Candida* spp. whereas, intracellularly located TLR7 and TLR9 participate in the recognition of the fungal nucleic acids that are released into TLR-containing vesicles during the digestion by phagocytes (77, 79).

In addition to the exogenous PAMPs, TLRs can be activated also by endogenous signals, such as damage-associated molecular patterns (DAMPs) released from dead and dying cells (80). The presence of DNA or RNA anywhere other than the nucleus or mitochondria is perceived as a DAMP and are sensed by intracellular TLRs. Inappropriate TLR signalling stimulated by extrinsic PAMPs and self-DAMPs holds the potential to activate uncontrolled activation of self-reactive B- and T-cells which induce autoimmunity assisted by the cells of the innate immunity (80).

Fig. 2. TLR1-10, ligands and signalling pathways (63, 70). Modified from Kumar *et al.* 2009 (63).



4.3.1.3 TLR signalling

The engagement of TLRs by microbial components triggers the activation of signal cascades, leading to specific immunological responses (77). After ligand binding, the intracellular TIR-domain binds to a single, or to a specific combination of recruited adaptor molecules, such as MyD88, TIRAP, TRIF and TRAM (77). All TLRs except TLR3 recruits MyD88 which leads to the activation of NF- κ B and mitogen-activated protein (MAP) kinase and the induction of inflammatory cytokines (76). TLR3 and TLR4 (with the combination with TRAM) use TRIF to activate an alternative pathway leading to the activation of NF- κ B and IRF3 and the induction of type I interferons and inflammatory cytokine productions (77). TLR2 and TLR6 use also TIRAP as an additional adaptor molecule in addition to MyD88. Because of the complexity of the signal cascade, the TLR signalling pathway is categorized into MyD88-dependent and TRIF dependent pathways (76, 77). The ligand diversity of TLRs can be explained in part by the selective usage of these adaptor molecules (77). Stimulation of several TLRs leads to the activation of several transcription factors, such as NF- κ B and to the induction of a variety of genes for cytokines, chemokines, and co-stimulatory molecules which play essential roles in recruiting various inflammatory cells into the infection sites and activating the adaptive immune response later in infection (77).

4.3.1.4 Transcription factors

4.3.1.4.1 NF- κ B

Nuclear factor- κ B (NF- κ B) acts as a central mediator of immune and inflammatory responses. It is also involved in stress responses and regulation of cell proliferation and apoptosis (81). NF- κ B are present in cells in an inactive state and after activation and nuclear translocation it controls the expression of genes encoding immune and pro-inflammatory mediators, such as TNF- α , IL-1 β and leukocyte and vascular adhesion molecules, which further propagate and amplify the inflammatory response (82). Some of these pro-inflammatory mediators can also activate NF- κ B and this type of positive regulatory loop may exacerbate and perpetuate local inflammatory reactions (83). Based on the significance with innate and adaptive immunity and cellular processes such as cell survival, proliferation, migration, and invasion the NF- κ B activity is tightly regulated (62). Dysregulation at any stage in the NF- κ B activation pathways may result in chronic inflammation, autoimmunity, and cancer (62, 83). NF- κ B activation and

inflammatory cytokines has been demonstrated to play an important role also in oral lichen planus (OLP) (84).

The significance of NF- κ B activity in cancer is further supported by several previous studies indicating a functional link between NF- κ B and the tumour suppressor protein, p53 (85, 86). Since NF- κ B is predominantly activated by extrinsic stresses, such as presence of bacteria and viruses, p53 acts as a guardian against intrinsic stresses, such as DNA damage and deregulation of protooncogenes (87). The p53 and NF- κ B pathways negatively regulate each other and are deregulated in opposite directions in tumours (85, 86). This antagonistic relationship of these transcription factors reflects the opposite principles of the physiological responses against intrinsic and extrinsic cell stresses (85).

4.3.1.4.2 p53

The tumour suppressor protein p53 is a transcription factor that plays an important role in preserving the genomic integrity; it controls the cell cycle and apoptosis if the DNA damage cannot be repaired (62). p53 may also influence immune responses by regulating TLR expression and the response of TLRs to their ligands (88, 89). Under normal conditions p53 resides in the cytoplasm in an inactive form and in response to various cellular stresses, such as DNA damage, virus infection, oxidative stress, and oncogene activation, it translocates into the nucleus (90). In the nucleus, p53 binds to several specific DNA sites and regulate transcription of numerous responsive genes and allows the cell to respond adequately to the applied stress (85). Physiologically, p53 prevents damaged cells from proliferating which is important because damaged cells are more likely to contain mutations which could lead to the development of cancer (90). A healthy cell maintains p53 at low levels and its half-life is short while the inactive and mutated p53 remains for longer periods in the cell and leads to cellular damage (91). The p53 protein is the most frequently mutated tumour suppressor in cancer and mutations of the TP53 gene can be found in approximately half of all human tumours (90). This may indicate that p53 plays a crucial role in preventing malignant transformation (90). In turn, overexpression of p53 has been associated with oral lichen planus (OLP) (91).

4.4 ORAL LICHENOID DISEASE

Oral lichenoid disease (OLD) encompasses oral lichen planus (OLP) and oral lichenoid lesion (OLL) which are chronic mucocutaneous inflammatory disorders of unknown aetiology (92, 93). Also, the term oral lichenoid reaction (OLR) is used for lesions that are like OLP. However, both OLL and OLR lack some of the clinical and/or histopathological features of OLP (94). The majority of OLP and OLL patients report a burning sensation or pain when eating or swallowing hot or spicy food that affects their quality of life (95). Most cases of symptomatic OLP are associated with erythematous and ulcerative lesions (96). Since there is no curative treatment for OLP the aim of current therapy is to eliminate mucosal erythema and ulcerations and alleviate symptoms (95). The improvement and control of oral hygiene should be a primary consideration in the management of OLP (97). In addition, mechanical trauma caused by badly fitting dentures or sharp filling margins or rough surfaces of dental restorations should receive attention (95).

Topical corticosteroids are used most commonly for the treatment of OLP. Topical cyclosporine, topical tacrolimus, or systemic corticosteroids may be indicated in patients whose condition is unresponsive to topical corticosteroids (98). The most important complication in OLP and OLL patients is the malignant transformation of the lesion even though the exact mechanism has not been clarified (99). However, regular follow-up for these patients is recommended (100).

4.4.1 Oral lichen planus

The prevalence of OLP is 0.5–4% depending on the population studied. It affects women more commonly than men and occurs mostly between 30 and 60 years of age (101). OLP is most commonly involved on the buccal mucosa (up to 90%), gingiva, dorsum of the tongue, labial mucosa, and lower lip (102). The clinical criteria for OLP issued by the World Health Organisation (WHO), indicates that OLP presents with multiple lesions in a bilateral and roughly symmetric distribution with presence of slightly raised grey-white lines (103)(Table 1). OLP has a wide range of clinical appearances that correlate with disease severity; reticular, erosive and, plaque-like are the most common ones and the ulcerative and bullous types are less common (104, 105).

The histology of OLP is characterized by the presence of a bandlike subepithelial infiltrate of inflammatory cells, predominantly T-lymphocytes within the epithelium and adjacent to damaged basal keratinocytes (93). In addition, the OLP

lesion shows degeneration of basal cells, disruption of the anchoring elements (hemidesmosomes, filaments and fibrils), and changes in the basement membrane that comprise breaks, branches, and duplications (106). Also, parakeratosis, acanthosis and “saw-tooth” rete peg formation are typical findings in OLP (106).

The precise cause of OLP is unknown. However, current data suggest that OLP is a T-cell-mediated autoimmune disease in which cytotoxic CD8+ T-cells trigger apoptosis of oral epithelial cells (106). During the initial phase CD8+ T-cells may recognize a self-peptide antigen expressed in association with the human leucocyte antigen (HLA) class I histocompatibility complex on lesional keratinocytes making lichen planus a true autoimmune disease (106). Alternatively, the antigen can be presented by antigen-presenting cells (APC), including Langerhans cells or keratinocytes in association with HLA class II histocompatibility complex to CD4+ T-cells (107). In the pathogenesis of OLP, it is likely that antigen presentation to both CD8+ and CD4+ T-cells is required to generate CD8+ cytotoxic T-cell activity (107).

An early event in lichen planus lesion formation may be keratinocyte antigen expression only at the future lesion site induced by different external or internal agents (106). This in turn, may alter the basal keratinocytes making them susceptible to apoptosis by cytotoxic T-cells (106). Such agents may be systemic drugs (lichenoid drug reaction), contact allergens in dental restorative materials or toothpastes (contact hypersensitivity reaction), mechanical trauma, viral or bacterial infection that induces the heat shock protein (HSP) antigen expression presented by keratinocytes (93). Thus, keratinocyte HSP expression in OLP may be an epiphenomenon associated with pre-existing inflammation caused by microbes (107). Also, other aetiological factors believed to be associated with OLP, such as genetic predisposition, stress, diabetes, and hypertension (93, 102).

Table 1. Modified WHO diagnostic criteria of oral lichen planus (OLP) and oral lichenoid lesions (OLL) (103, 108).

Clinical criteria	
	Presence of bilateral, roughly symmetrical lesions Presence of reticular pattern; a lace-like network of slightly raised gray-white lines In the presence of reticular lesions elsewhere in the oral mucosa, erosive, plaque-like, bullous, and atrophic lesions are accepted as a subtype In all other lesions that resemble OLP but do not complete the above criteria, the term “clinically compatible with” should be used
Histopathologic criteria	
	Band-like zone of cellular infiltration that is confined to the superficial part of the connective tissue, predominantly lymphocytic infiltration Liquefaction degeneration of basal cell layer Absence of epithelial dysplasia When the histopathologic features are less obvious, the term “histopathologically compatible with” should be used
Final diagnosis OLP or OLL	
	To achieve a final diagnosis both clinical and histopathologic criteria should be included
OLP	A diagnosis of OLP requires fulfilment of both clinical and histopathologic criteria
OLL	The term OLL will be used under the following conditions:
	<ol style="list-style-type: none"> 1. Clinically typical of OLP but histopathologically only “compatible with” OLP 2. Histopathologically typical of OLP but clinically only “compatible with” OLP 3. Clinically “compatible with” OLP and histopathologically “compatible with” OLP

4.4.2 Oral lichenoid lesion

The oral mucosa also manifests lichenoid lesions (OLL), such as hyperkeratotic, white, thickened, inflammatory reactions, which are most commonly considered as an immunopathological reaction to various aetiological factors, such as systemic drug exposure and local contact hypersensitivity against dental restorative materials like amalgam (102, 105). Despite of the distinct aetiopathological features, OLP and OLL are histologically indistinguishable and therefore the diagnosis is based on both clinical and histological findings (104). Since both conditions possess overlapping clinical and histopathological features,

similar therapies may be used in OLP and OLL (105). However, unlike OLP, OLL resolves after elimination of the causative agent (105).

4.4.3 Malignant transformation

Both OLP and OLL are classified as potentially malignant disorders (100, 108). According to the latest meta-analysis the frequency of malignant transformation in OLP ranges from 0,5% to 1.3% and in OLL from 1,2% to 4,9%, respectively (109). The average time from the diagnosis to the malignant transformation is 51,4 months (100). In OLP the highest malignant transformation rate noted is in erosive lesions and the most common site of malignant transformation was the tongue (30%), followed by the buccal mucosa (20%) and gingiva (17%) (109). Malignant transformation is still controversial due to the lack of universally accepted specific clinical diagnostic criteria of OLP and further prospective studies are required (102, 110). However, it has been suggested that the oral mucosa affected by OLP may be compromised to the extent of being more sensitive to exogenous mutagens in alcohol, tobacco, and microbes (96). Alternatively, the chronic inflammatory response and simultaneous mucosal wound healing response in OLP may increase the likelihood of cancer-forming gene mutations (96). This hypothesis was supported by findings which showed that macrophage migration inhibitory factor (MIF) released from T-cells and macrophages suppresses the transcriptional activity of the p53 (111). Cellular stress, such as DNA damage, can lead to activation of p53 that play an important role in preserving the genomic integrity (62). An association between overexpression of p53 and chromosomal alterations has been shown in OLP (91).

4.4.4 TLR and NF-κB in OLD

As mentioned before, stimulation of several TLRs leads to the activation of several transcription factors, such as NF-κB and dysregulation at any stage in the NF-κB activation pathways may result in chronic inflammation, autoimmunity, and cancer (62, 112). Still the function of TLRs and NF-κB in OLP remains unclear (84, 113). Keratinocytes in OLP lesion show an increased NF-κB activity which is correlated with the recruitment of numerous cytotoxic cells in OLP (84). The degree of NF-κB activation in OLP has been suggested to correlate with the severity of the disease (84). In previous literature on TLR and OLD, several TLRs expression, specially TLR1, TLR2, TLR4 and TLR9 were shown to be increased in the lesions compared to the healthy oral mucosa (114-120) (Table 2). In addition,

soluble forms of TLR2 and TLR4 were found to be increased and functional in saliva in OLP patients (71, 73).

Table 2. The expression several TLRs has been shown to be increased in OLP compared to the healthy oral mucosa. IHC: Immunohistochemical staining; RT-PCR: real-time PCR; sTLR: soluble TLR; IF: immunofluorescence; WB: western blot; FCM: flow cytometry; ↑ and ↓: up- and downregulation; ±: no differences between the groups.

TLR	Disease	TLR studied	Sample	Method	Reference
TLR2↑	OLP	TLR2	peripheral blood mononuclear cells	IHC, RT-PCR, WB, ELISA	(121)
TLR4↑	OLP	TLR4	Biopsies	IHC, IF	(122)
TLR7↑, TLR8↑, TLR9↑	OLP	TLR7, TLR8, TLR9	Biopsies	IHC, RT-PCR	(118)
TLR4↑	OLP	TLR4	Biopsies	IHC, RT-PCR, WB	(123)
TLR2 polymorphism were associated with OLD	OLD	TLR1, TLR2, TLR4, TLR6, TLR9, TLR10	Mouthwash samples, DNA was extracted from the buccal cell pellet	Pyro-sequencing	(124)
TLR1↑, TLR2↑, TLR4↑, TLR7↑, TLR8↑, TLR9↑	OLR	TLR1-10	Biopsies	IHC, RT-PCR	(119)
TLR4↑	OLP	TLR4	Biopsies and cell culture	IF	(125)
TLR4↑	OLP	TLR4	Cell culture	RT-PCR	(126)
TLR2±, TLR3↓, TLR4↑, TLR8±	OLP	TLR2, TLR4, TLR8	Biopsies	IHC, RT-PCR	(117)
TLR1↑	OLP and OLR	TLR1	Brush cytology samples	IHC, RT-PCR	(120)
TLR3 polymorphism were associated with OLP	OLP	TLR2, TLR3, TLR4	Biopsies	RT-PCR	(127)
TLR2 ±	OLP	TLR2	Saliva and blood samples	RT-PCR	(128)
TLR4↑	OLP	TLR4	Biopsies	IHC, RT-PCR	(113)
TLR4↑, TLR9↑	OLP	TLR4, TLR9	Biopsies	IHC	(114)
TLR2↓, TLR4↑	OLP	TLR2, TLR4	Biopsies	IHC, RT-PCR	(115)
TLR2↑, TLR4↓	OLP	TLR1-10	Biopsies	IHC, RT-PCR, FCM	(116)
sTLR4↑	OLP	sTLR2, sTLR4	Saliva samples	WB	(73)
sTLR2↑	OLP	sTLR2	Saliva samples	WB	(71)

Pubmed search: (((("olp") OR "oll") OR "olr")) AND (("tlr") OR "toll like receptor"))

4.5 ORAL SQUAMOUS CELL CARCINOMA

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor in the oral cavity and accounts for more than 90% of all oral cancers (129). There is much geographical variation regarding mortality rates and incidence which is increasing in many parts of the world despite all the advances in modern medicine (129). According to the latest reports of the International Agency for Research on Cancer (IARC) for oral cancer, including lips and oral cavity, annual estimates of age standardized incidence and mortality are 5,5/100 000 and 2,7/100 000 in men and 2,5/100 000 and 1,2/100 000 in women, respectively (129). In Finland in 2015 there were over 410 new cancers of lip, tongue and oral cavity cancer and the mortality rates were over 140 in both sexes (130). Regardless of advances in surgical techniques the five-year overall survival rate in Finland for OSCC of the tongue remains 47% (131). The mean age at diagnosis for oral cancer is 60 years in men and 67 years in women (132). There is substantial evidence that early diagnosis would reduce the morbidity and mortality from oral cancer (48).

4.4.5 Risk factors

Tobacco (also smokeless) and chronic alcohol consumption are the two most important known risk factors for the development of OSCC. They have been shown to have a synergic effect (133). It has been estimated that smoking causes over 85% of deaths caused by oral cancer (134). In addition, poor oral hygiene with smoking and simultaneous alcohol consumption have been associated with increased risk of oral cancer in several studies (42, 47, 48). Other possible risk factors for OSCC include chronic infections, viral infections, such as HPV, immunodeficiency, UV radiation, dietary factors, and precancerous lesions, such as erythroplakia and leucoplakia (62, 135). OSCC is a multifactorial disease with no single clearly recognizable cause. However, it has been estimated that 75% of all oral cancers could be prevented by the elimination of risky lifestyles such as tobacco smoking and alcohol consumption and by protecting against solar irradiation (136).

OSCC develops over many years and during this period epithelial cells are affected by various mutagens, especially alcohol and tobacco (48). Oncogenesis is a progression from a normal healthy cell to a pre-malignant or a potentially malignant cell, where several DNA mutations occur leading to loss of growth control and eventually the ability to proliferate autonomously (48). One of the fundamental concepts of the genetic mechanisms behind cancer is the

overexpression of oncogenes and/or the silencing of tumour suppressor genes, such as p53 (90).

4.4.6 Bacteria and yeasts on OSCC lesion

Infection is one of the most important causes of cancer and almost one in every five malignancies can be attributed to infectious agents (137). Several bacterial species have been associated with different cancers. For example, *Chlamydia trachomatis* infection has been associated with an increased risk for the development of invasive cervical carcinoma (138). Bacteraemia and endocarditis due to *Streptococcus bovis* have likewise been linked with malignancies in the colon (139). *Helicobacter pylori* infection has been considered a causative agent of both gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphomas (140). The association of microbes with OSCC is of increasing interest. Emerging evidence suggests a link between chronic periodontal disease and oral cancer and variety of periodontal bacteria such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Prevotella intermedia*, are related to OSCC (141). It has been demonstrated that surface biofilms in oral carcinoma harbour significantly increased numbers of aerobes and anaerobes as compared to the healthy mucosa surface on the same patient (12, 47, 51). The results of our study group also support this notion. Likewise, there are differences in colonisation of *Candida albicans* on OSCC lesion compared to the healthy site but it is still uncertain and debatable whether microbial invasion is a causal or secondary event in oral premalignant and malignant lesions (48, 51).

There are several mechanisms by which different microbes may play a role in cancer development. It has been proposed that microbes affect mucosal cells through the induction of chronic inflammation (62), by interfering, either directly or indirectly, with eukaryotic cell cycle and signalling pathways (142), or via the metabolism of potentially carcinogenic substances, acetaldehyde (36, 38, 39). Several bacteria and *Candida* strains in the mouth can produce carcinogenic acetaldehyde from alcohol which may explain why poor oral hygiene is often associated with oral cancer in heavy drinkers and smokers (48, 143). One of the molecular pathogenesis of oral cavity cancer is the inactivation of tumour suppressor p53 (90).

Recent research has provided us considerable amounts of information regarding the microbial mechanisms purported to cause oral cancer. However, it is still debatable whether microbial infections initiate cancer, or is it the preexisting

cancer that compromises the host's immunity followed by secondary microbial colonization (144). In addition, a debatable question is that, would certain bacteria in saliva or on the OSCC lesion be of any estimable value in the diagnosis or treatment of oral cancer, respectively (145). Thus, to demonstrate a role for microbes in the development of OSCC or OLD, the first step must be to identify such organisms on the lesion. This emphasises the importance of the correct sampling method and sampling site for the analysis of lesion specific microbes.

4.4.7 Treatment

Surgery, radiotherapy, and chemotherapy are three primary approaches to cancer treatments and during these treatments the oral cavity goes through radical changes (146). Surgical excision of the tumour often results in considerable lack of tissue, and pedicled flaps or free tissue transfers of bone, skin and muscle are used for reconstruction. Radiotherapy to the primary tumour site and regional lymph nodes, as a pre- or postoperative treatment are given to patients with aggressive and large tumours and ones at risk for metastases. Radiotherapy usually starts as soon as the primary healing of the operation wounds has completed (146). The combined chemotherapy with radiotherapy has an 8% effect on the 5-year overall survival in head and neck cancer (147).

For most patients, anticancer therapy, irradiation, chemotherapy, or surgery results in permanent damage to their salivary glands and lifelong xerostomia. In addition, the increase of keratinised surfaces when skin-lined microvascular flaps are used alter the micro-environment of the oral cavity (148). Thus, anticancer therapy compromises the defence mechanism of the oral mucosa and is accompanied by a proliferation of the mucosal biofilm with an overgrowth of yeast and bacteria (144). Lack of saliva and changes in oral surfaces making them more susceptible to heavy yeast colonization cause a lifelong high risk for oral candidosis for these patients (20). In fact, cancer lesions itself might even increase the local and systemic infection risk in oral cancer patients, even before specific tumour treatment (144).

To prevent or treat oral mucositis in patients receiving radiation and/or chemotherapy a regular use of oral care protocols consisting of brushing, flossing, rinsing, and moisturizing, are important (149). The post-operative antimicrobial treatment should be targeted against pathogens which should be identified using a reproducible sampling method. Traditional sampling methods, e.g. mouth rinses, saliva culture or tongue scrapings, are often impossible to perform due to the

xerostomia and changes after surgical treatments and may result in false negative results. This is especially problematic, as clinical symptoms may be non-existent due to neural damage and to the decrease in blood flow in the irradiated and reconstructed tissues (20). The optimal site and method of sampling for oral microbes in oral cancer patients is not known.

5 AIMS OF THE STUDY

The objectives of this thesis were to investigate how the method and site of microbial sampling affect the discovery of oral microbial flora on OSCC lesions. Secondly, to explore the ability of lesion specific oral microbes to produce acetaldehyde in OLD and OSCC patients using a quantitative sampling method. Furthermore, to investigate the immunohistochemical expression and tissue localization of TLR, p53 and NF- κ B in mucosal biopsies from patients with OLD.

The specific aims were as follows:

- I. To investigate how the sampling method and site affect the discovery of *Candida* species from the oral cavity in OSCC patients.
- II. To develop a site-specific and easy-to-use sampling method that would give representative and quantitative results for samples from the oral mucosa.
- III. To explore the lesion specific microbial flora in OLD and OSCC patients using a site-specific and quantitative sampling method and to explore the ability of these microbes to produce acetaldehyde when exposed to clinically relevant levels of ethanol.
- IV. To compare the immunohistochemical expression levels and tissue localization of TLR1–10, p53 and NF- κ B in mucosal biopsies from patients with OLD and healthy controls.

6 MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 Subjects and study design (I-IV)

Study I: Eighteen previously untreated patients with primary oral cancer were enrolled in the study (Table 3). All patients were hospitalized due to oral cancer treatment during 2004–2005 (mean age 60 years, range 42–81, female:male ratio 7:11). Five non-medicated volunteers of the hospital personnel were included as healthy controls (mean age 42 years, range 28–54 years, female:male ratio 2:3). For this study, five patients were examined prior to all cancer treatment and thirteen patients were examined 2–4 weeks ($n = 5$) or 8–12 weeks ($n = 8$) after the primary surgical treatment. From the thirteen patients who had undergone surgery, two received chemoradiotherapy and eleven received conventionally fractionated radiotherapy (mean total dose of 55 Gy; range 20–76 Gy) post-operatively. The primary sites of the oral cancer were the tongue ($n = 6$), buccal mucosa ($n = 1$), mandible ($n = 6$) and maxilla ($n = 2$). In three cases, metastasis had been identified. The general status of the dentition and dental status was recorded according to the WHO Diseased Missing Filled (DMF) Index. The oral hygiene (examiner-assessed subjective scale 1–3), as well as the use of antifungals, was recorded.

Table 3. Subjects of the first study.

	Number of patients			Total	Controls
	Pre-operative	2-4 weeks post-operative	8-12 weeks post-operative		
Total number	5	5	8	18	5
Female:male	1:4	2:3	4:4	7:11	2:3
Age (years)					
Mean	61	57	62	60	42
Range				42-81	28-54
Location of the cancer					
Tongue	1	0	4	5	
Buccal mucosa	1	0	0	1	
Floor of the mouth	1	3	3	7	
Maxilla	0	1	1	2	
Metastasis	2	1	0	3	
Treatment					
Surgery	0	5	8	13	
Radiotherapy	0	3	8	11	
Mean dose				54 Gy	
Dose range				20-76 Gy	
Chemoradiotherapy	0	1	1	2	

Study II: From the staff of the Department of Bacteriology and Immunology of Helsinki University a total of fourteen non-medicated healthy volunteers with good oral health, were enrolled in the study (mean age 36 years, range 27–50, female:male ratio 7:7). The subjects were not receiving any systemic or topical antimicrobial treatment at the time of sampling or during the previous three months. The volunteers were asked not to consume any food for 1 hour prior to the sampling.

Study III and IV: A total of 90 patients, 30 with newly diagnosed primary oral squamous cell carcinoma (OSCC), 30 with oral lichenoid disease (OLD) and 30 healthy controls treated at the Department of Oral and Maxillofacial Surgery, Helsinki University Central Hospital or at the Helsinki University Dental Hospital

during 2007–2011 were enrolled (Table 4). For the third study, microbial samples were collected from all three patient groups and for the fourth study, surgical biopsies were collected from OLD and control groups. Patients potentially suitable for enrolment were identified from weekly theatre list by the research team member and the exclusion criteria were antimicrobial therapy (i.e. antibiotics, antifungals, or antiviral agents) within the past seven days and HIV or hepatitis virus infection. All study participants were generally well without any systemic diseases or immune suppression predisposing them to infection.

Patient questionnaire. The subjects filled in a modification of the World Health Organization Alcohol Use Disorders Identification Test (WHO AUDIT) questionnaire including open and closed questions about their drinking and smoking habits (150). Approximated daily and weekly amounts of consumed alcohol and tobacco were recorded, and the consumption were based on self-reporting. Patients who smoked regularly were defined as smokers. A member of the research team gave the forms to the participants and was available in case of any questions.

Patients with OSCC. Thirty patients with clinically and histopathologically diagnosed OSCC were enrolled. The anatomical sites of the cancerous lesions were the tongue ($n = 9$), the gingiva ($n = 10$), the sulcus ($n = 2$), the floor of the mouth ($n = 5$), the palate ($n = 3$), and the tonsil ($n = 1$).

Patients with OLD. Thirty patients were enrolled into the study with the clinical diagnosis of OLD from which twenty-four cases were histologically confirmed as oral lichen planus (OLP; $n = 10$) or lichenoid reaction or lichenoid lesion (OLR or OLL; $n = 14$). The anatomical sites of the OLD lesions were the tongue ($n = 7$) and the buccal mucosa ($n = 17$).

Healthy controls. Thirty generally healthy individuals, which were patients referred to the Department of Oral and Maxillofacial Surgery for operative wisdom tooth extraction were included as healthy controls. Healthy control patients had no clinically evident mucosal lesions in the oral cavity.

Table 4. Subjects of the third and fourth study.

	OSCC	OLD	Controls
Total number	30	24	30
Female: male	12:18	16:8	19:11
Age in years (range)	65.6 (39-85)	54 (24-74)	30.4 (19-56)
Smokers	9 (32%)	4 (19%)	9 (31%)
Female: male	2:7	2:2	5:4
Non-drinkers	6 (21%)	2 (10%)	3 (10%)
Alcohol consumers	23 (79%)	19 (91%)	26 (90%)
Female: male	8:15	15:7	16:11
Heavy drinkers	5 (17%)	1 (5%)	2 (7%)
Female: male	0:5	0:1	2:0
Non-responders	1 (3%)	3 (13%)	1 (3%)
Location of the lesion			
Tongue	9	7	
Buccal mucosa	0	17	
Gingiva	10	0	
Sulcus	2	0	
Floor of the mouth	5	0	
Palate	3	0	
Tonsil	1	0	

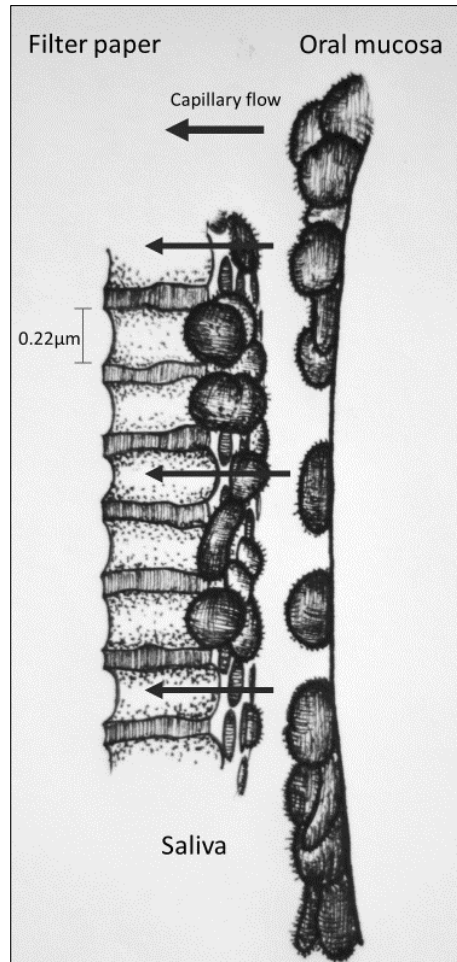
6.2 METHODS

6.2.1 Sampling methods (I, II and III)

Study I: For culture of yeasts, eighteen oral cancer patients and five control subjects were sampled once semi-quantitatively from the labial sulcus, saliva, dental plaque, and dorsum of the tongue. All samples were taken non-invasively with sterile instruments and cotton swabs and care was taken to perform the sampling in a standardized way and to avoid contamination from adjacent areas. The precise site of sampling varied a little from patient to patient, depending on the dentate status and anatomical circumstances in the mouth due to the anatomical changes after surgical treatment. For the labial sulcus sample, each sulcus was gently swabbed with single swipes and the saliva sample was collected by placing the swab into a moist area in the floor of the mouth for 10 s. The dental plaque sample was taken from the labial surface of one lower molar tooth using a gingival probe. Samples from the dorsum of the tongue were taken with one gentle scrape using a spatula.

Study II: Two site-specific non-invasive sampling methods for microbiological analyses of the healthy oral mucosa were compared. The samples were obtained using a filter paper and swab using a standardized procedure as far as possible. The filter paper sampling method was developed for this study. Samples from adjacent areas on buccal mucosa for each subject were collected consecutively in the following order, i.e. swab sample and filter paper imprint sample. For the swab sample an area of diameter approximately 13 mm, estimated using a template, was rubbed with a dry and sterile swab (Copan Diagnostics, Corona, USA). For the filter paper sample, a hydrophilic mixed cellulose ester MF-Millipore Membrane filter (GSWP01300; Millipore Inc., MA, USA, pore size 0.22 μm , diameter of 13 mm) was placed gently on the buccal mucosa for 30 s, with the glossy side of the filter paper placed against the mucosa (Figure 3). The optimal time for the filter paper sampling method was based on a pilot study.

Fig. 3. Schematic illustration of the filter paper. The pore size ($0.22\ \mu\text{m}$) of the hydrophilic filter paper allows capillary flow of saliva into the filter paper creating a gentle suction and thereby releasing adherent microorganisms without rubbing. The filter paper sampling method was developed for the study II.



Study III: After clinical assessment, microbial samples for microbiological analyses and acetaldehyde measurement were obtained using the filter paper sampling method described in the study II. In OSCC and OLD patient groups two samples were collected from each patient: one from a representative mucosal lesion and another from a clinically healthy contralateral site. Samples from the healthy controls were obtained from the buccal mucosa. Sampling methods and sites in study I, II and III are shown in table 5.

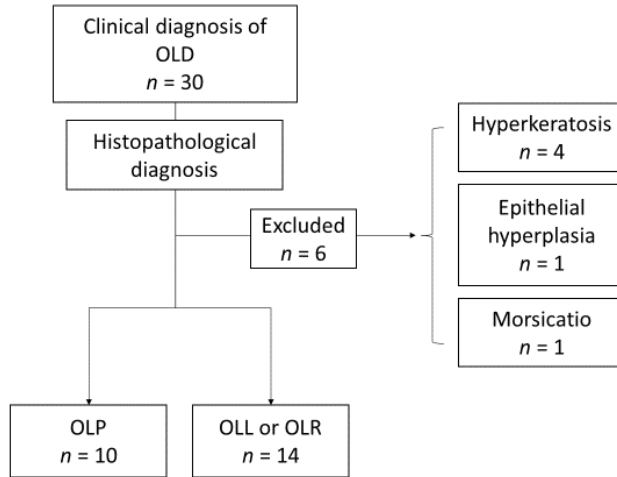
Table 5. Sampling methods and sites in study I, II and III.

Sampling tool	Sampling method	Sampling site	Study I	Study II	Study III	
					Mucosal lesion	Contralateral healthy site
Swab	Gently swipe	Sulcus	X			
		Saliva	X			
		Buccal mucosa		X		
Gingival probe	Gentle scrape	Lower molar	X			
Spatula	Gentle scrape	Dorsum of the tongue	X			
Filter paper	Placing on the mucosa	Buccal mucosa (II)		X		
		Oral mucosa (III)			X	X

6.2.2 Collection of histopathological samples (IV)

As part of routine histopathological diagnostics full thickness biopsies including epithelial and stromal tissue were collected from the site of active disease process of OLD patients. The samples were fixed in 10% buffered formalin and embedded in paraffin. The diagnoses of OLP, OLL or OLR were based on the clinical and histopathological criteria provided by the World Health Organization (108) and clarified by van der Meij (151). Of the 30 patients enrolled into the study with the clinical diagnosis of OLD 24 were histopathologically confirmed as OLP ($n = 10$) or OLL/OLR ($n = 14$). The remaining six samples were diagnosed as hyperkeratosis ($n = 4$), epithelial hyperplasia ($n = 1$) and morsicatio ($n = 1$) and were excluded from the analyses (Figure 4). The biopsies from healthy control patients were taken from the non-inflamed, healthy buccal mucosa at the incision site immediately after surgical extraction of a retained wisdom tooth.

Fig. 4. Patients in the study IV. Of the 30 patients enrolled into the study with the clinical diagnosis of OLD, 24 were histopathologically confirmed as OLP ($n = 10$) or OLL/OLR ($n = 14$). The diagnoses were based on the clinical and histopathological criteria provided by the World Health Organization and clarified by van der Meij (108, 151).



6.2.3 Culture (I, II and III)

For the identification and culture of yeasts and bacteria the microbiological samples were immediately taken to the laboratory, Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, and all samples were cultured within one hour.

Study I. For the culture and identification of yeasts the samples were collected into sterile tubes containing 0,5ml sterile saline and after vortexing 100µl of the saline was plated onto Sabouraud dextrose plates (SP; Sabouraud Dextrose Agar [Lab M], Bacto Agar [Difco Laboratories, Basel, Switzerland] supplemented with penicillin [100,000 iu/ml] and streptomycin) and incubated at 37°C for 48h. Thereafter, colonies were further counted and cultivated on CHROMagar Candida medium (CHROMagar) for the identification of *Candida* species. The Bichro-Dubli latex co-agglutination test (Fumouze Diagnostics) was used to differentiate between *C. albicans* and *C. dubliniensis* and species other than *C. albicans* and *C. dubliniensis* were identified by API 32C auxanographic strips (bioMérieux) (Figure 5). Multiple colonies were tested at every identification step.

Study II and III. The samples were collected into sterile tubes containing 5ml sterile saline and mixed for 30s with five sterile Ø3 mm glass beads. Samples were further diluted 10-fold and 100µl of the dilution were cultured on selective and nonselective media under aerobic and anaerobic conditions to detect and enumerate:

1. yeasts

Sabouraud dextrose plates (SP; Sabouraud Dextrose Agar [Lab M], Bacto Agar [Difco Laboratories, Basel, Switzerland] supplemented with penicillin [100,000 iu/ml] and streptomycin) was used.

2. total cultivable bacteria

Fastidious anaerobe agar (FAA; Fastidious Anaerobe Agar; LAB 90 [Lab M, Lancashire, UK] supplemented with 5% horse blood) was used.

3. total aerobic bacteria

Lysed blood agar (BA; Trypticase soy agar [BBL 211047; BD Diagnostics, Franklin Lakes, NJ, USA] and Mueller Hinton agar [BBL 212257; BD Diagnostics] supplemented with 5% horse blood) was used.

4. anaerobic gram-negative bacteria

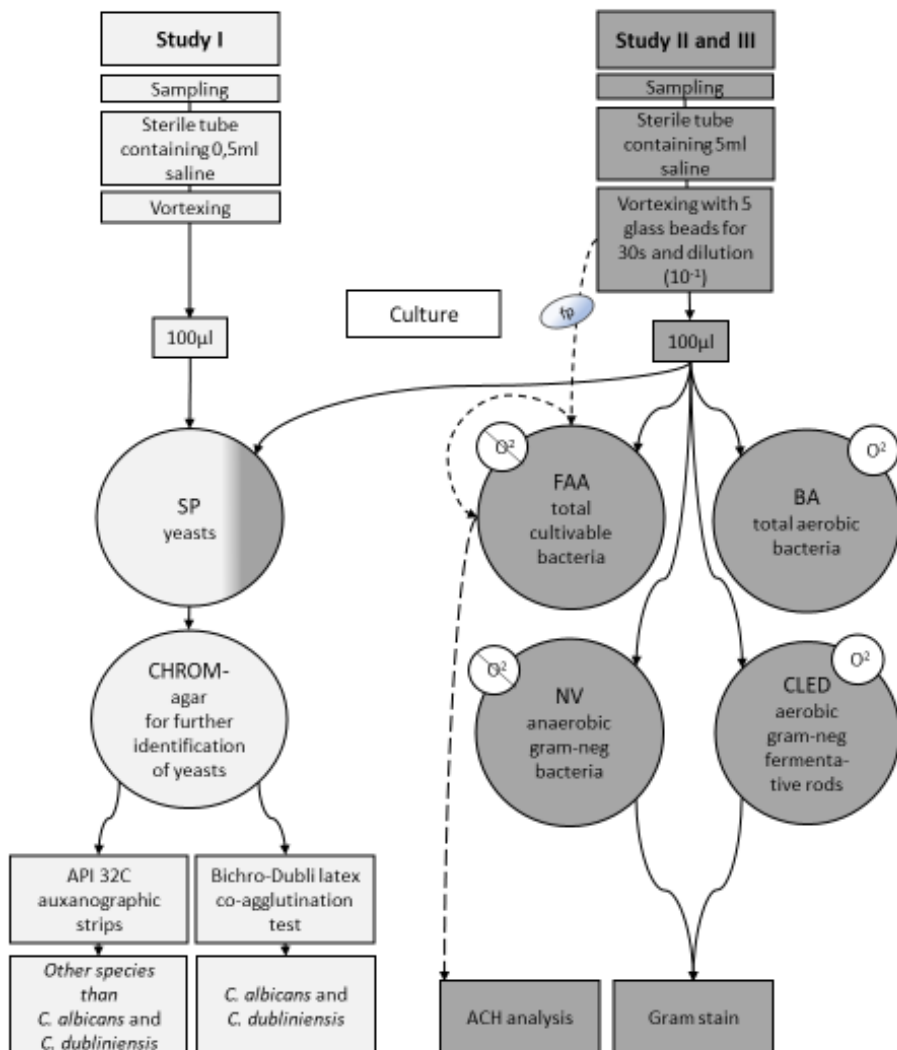
Neomycin-vancomycin blood agar (NV; blood agar and neomycin sulfate [Sigma N1876; Sigma-Aldrich, St. Louis, MO, USA] supplemented with vancomycin [7.5 m/ml], menadion [0.5 mg/ml] and sheep blood 5%) was used.

5. aerobic gram-negative fermentative rods

Cysteine-, lactose- and electrolyte-deficient agar (CLED; C.L.E.D. medium [BBL 212218; BD Diagnostics]) was used.

FAA and NV plates were incubated under anaerobic conditions at 37°C for seven days and BA, CLED and SP plates were incubated at 37°C for two days. For the acetaldehyde analyses, both sides of the filter paper were placed onto an FAA plate for 30s and plates were evenly streaked and incubated as described above. After incubation, the numbers of bacteria and yeasts were enumerated [colony forming units (CFU)]. Gram stain was performed on all different colony morphology types from NV and CLED agars and the number of gram-negative colonies was enumerated. The ratio of aerobic to anaerobic bacteria and the ratio of gram-negative to gram-positive bacteria were determined. (Figure 5)

Fig. 5. Schematic illustration of the culture and identification of yeasts and bacteria in study I, II and III. BA and CLED were incubated in aerobic conditions (O_2) and FAA and NV plates under anaerobic conditions (Θ^2). For the acetaldehyde (ACH) analyses both sides of the filter paper (fp) were placed directly onto an FAA plate for 30s.



6.2.4 Acetaldehyde analysis (III)

Microbial colonies on the FAA plate were carefully scraped and washed off with 3ml of sterile saline solution and 400µl of the solution was transferred into parallel gas chromatograph vials. Then 50µl of phosphate buffered saline containing ethanol (final concentration 22 mM) was added, after which the vials were sealed immediately, and the samples were incubated for 1h at 37°C. The reactions were ended by injecting 50µl of 6M perchloric acid (PCA) through the rubber septum of the vial. Control vials in which perchloric acid was added prior to ethanol were used to measure background acetaldehyde and ethanol levels. Three parallel samples were analysed, and the mean values were used for statistical analysis. The formed ACH levels were measured by gas chromatography (Perkin Elmer Headspace Sampler HS 40XL, Perkin Elmer Autosystem Gas Chromatograph equipped with Ionization Detector FID, Waltham, MA, USA) (152).

6.2.5 Immunohistochemical staining (IV)

Sixty tissue sections (30 OLD and 30 control sections) were prepared for the histopathological diagnosis and immunohistochemical analyses. Tissue sections, 4µm in thickness, were prepared from the paraffin embedded samples and applied to glass slides followed with deparaffination in xylene and rehydration in graded ethanol. The sections were incubated in pepsin for 30min at room temperature to expose the antigenic determinants after formalin fixation and paraffin embedding. Endogenous peroxidase activity was quenched in the sections by incubating in hydrogen peroxidase in methanol.

TLRs. The optimal primary antibody concentrations for immunohistochemistry was selected based on a pilot study. The final IgG concentrations of the polyclonal anti-human antibodies used in the study IV are shown in the table 6. Control incubations were performed by replacing primary antibodies with protocol buffer. Sections from each sample were also stained with periodic acid-Schiff (PAS) to determine the presence or absence of candida species. The TLRs were visualized using avidin-biotin-peroxidase complex method (catalogue nos., PK-4001 and PK-4005; Vectastain ABC kit; Vector Laboratories, Peterborough, England).

NF-κB and p53. For the immunohistochemical staining with NF-κB, the tissue sections were buffered in citrate, pH 6 and heated for 10min in microwave oven and incubated for 1h in room temperature with an optimally diluted NF-κB antibody. For the immunohistochemical staining of p53, the tissue sections were

buffered in Tris-EDTA, pH 9 and heated 15min in microwave oven and incubated for 30min RT with an optimally diluted p53 antibody. The concentrations of NF- κ B and p53 IgG antibodies used in this study are shown in the table 6. After the primary antibody incubation, the tissue sections were incubated separately with Dako REAL™ EnVision™ kit using Dako automated immunostaining instruments. The reactions were visualized by Dako REAL™ DAB+ Chromogen also included in the kit (catalogue number K5007, Dako Glostrup Denmark). Control incubations were performed by replacing primary antibodies with protocol buffer.

Gingival tissue samples from patients with chronic periodontitis obtained during periodontal flap operations were used in the pilot study and in the fourth study as positive controls for all immunohistochemical staining (153, 154).

Table 6. The optimal IgG concentrations of the polyclonal anti-human antibodies used in the study IV.

Primary antibody	Type	Dilution	Catalogue nr.
TLR1	polyclonal rabbit IgG	1:50	sc-30000*
TLR2	polyclonal rabbit IgG	1:50	sc-8689*
TLR2	polyclonal goat IgG	1:50	sc-10739*
TLR3	polyclonal rabbit IgG	1:50	sc-10740*
TLR4	polyclonal rabbit IgG	1:50	sc-10741*
TLR5	polyclonal rabbit IgG	1:50	sc-10742*
TLR6	polyclonal rabbit IgG	1:50	sc-30001*
TLR7	polyclonal rabbit IgG	1:40	sc-30004*
TLR8	polyclonal rabbit IgG	1:50	sc-25467*
TLR9	polyclonal rabbit IgG	1:40	sc-25468*
TLR10	polyclonal rabbit IgG	1:40	sc-30198*
NF- κ B	polyclonal rabbit IgG	1:150	sc-114*
p53	monoclonal mouse IgG	1:600	M7001**

*=Santa Cruz Biotechnology, Santa Cruz, California, USA

**=Dako Glostrup Denmark

6.2.6 Microscopical analyses (IV)

The immunohistochemical expression for TLR1-TLR10, p53 and NF- κ B was analysed using a light microscope (Nikon Eclipse 80i). Results were scored semi-quantitatively and photographed using an attached camera (Nikon DS-Fi1). All samples and staining's were analysed and scored by four authors (Peter Rusanen, Jaana Hagström, Emilia Marttila and Tuula Salo) blinded for each other's scoring and clinical data and discrepancies were settled within the team. The staining quantity of the basement membrane (BM) zone and of the cells in the basal, intermediate, and superficial layers of the epithelium were scored in a four-point scale as follows:

0 = no staining

1 = staining of approximately 1-33% of cells or of the BM zone

2 = staining of 34-66% of cells

3 = staining of 67-100% of cells

6.2.7 Statistical methods

Data is presented as means (study I – IV), in standard error of mean (\pm SEM; study II – IV) and in standard deviation (study II). The statistical differences were analysed by using GraphPad Prism version 5.00 (GraphPad Inc. San Diego, California, USA; study II, III and IV). The Mann-Whitney test was used for the analysis of the colony morphology types on different agars (study II). The two-tailed Mann Whitney test and Spearman's rho (r_s) was used for the analyses of correlations and the Wilcoxon signed-ranks test was used to compare the differences between the different layers of samples (study III and IV). *P*-values of less than 0.05 were considered statistically significant.

6.2.8 Ethical considerations

The study protocol was approved by the ethical committee of the Helsinki University Central Hospital (study I-IV; study I: ethical permit number 525/E6/2003 28.01.2004; study II – IV: § 47/2007, 25.4.2007, Dnro 126/E6/07). All subjects signed an informed consent.

7 RESULTS

7.1 OPTIMAL SAMPLING SITE IN OSCC PATIENTS (I)

40% of the control subjects and the pre-operative groups had positive *Candida* growth. However, the colony density was found to be markedly higher in the OSCC patient group before the cancer treatment compared to the controls (Figure 6). After cancer treatment, the incidence was found to be increased and 69% (9/13) of the patients were positive for *C. albicans*. Of the patients who had undergone operations, 75% (6/8) were positive for *Candida* 8-12 weeks post-operatively (Figure 6). In addition to the increase of the incidence of *Candida*, the colony forming units (CFU) also increased after the beginning of the cancer treatment.

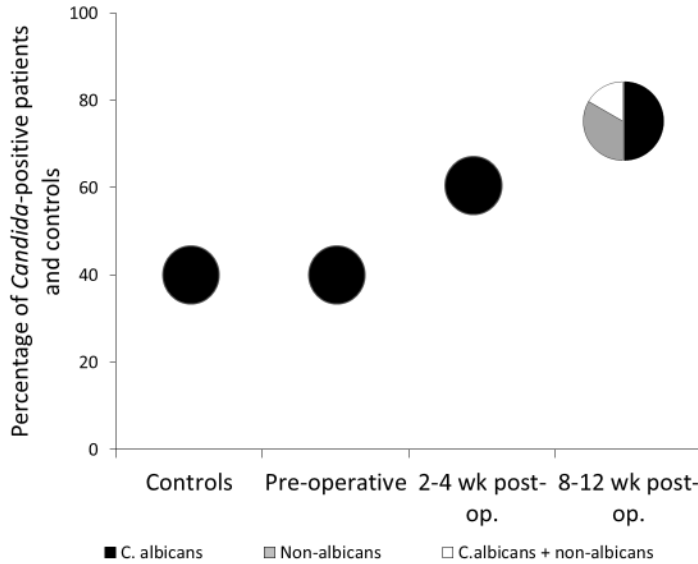
The most sensitive sampling site was found to be the labial sulcus, from which all *Candida* positive cases could be confirmed. However, the number of CFU was highest in the dental plaque samples. The samples from the dorsum of the tongue was found to be more sensitive than saliva in detecting *Candida* in the patients and in the healthy controls. In detecting the different species of *Candida*, all sampling methods were equally sensitive.

C. albicans was found to be the predominant species and it was the only yeast detected in the control group as well as in the patient groups before and 2-4 weeks after the cancer treatment. In the patients at 8-12 weeks after the cancer treatment, 50% of the *Candida*-positive patients, species other than *C. albicans* was identified (Figure 6). *C. dubliniensis* was not found in any of the patient samples. Antifungal prophylaxis, mainly fluconazole 100mg p.o. or 150 mg i.v. daily, had been given to 44% of the patients. About half of the patients who received antifungal treatment still had positive yeast growth, mainly of species other than *C. albicans*. Of the seven patients with negative yeast growth, four were receiving antifungal treatment. Of the patients undergoing radiotherapy, 67% had positive yeast growth, although 63% were receiving antifungal treatment. All patients receiving chemoradiotherapy had positive yeast growth.

Oral hygiene and the general status of the dentition of the patients was recorded. Of the patients, ten were smokers from which seven had positive yeast growth. A hospital dentist had seen all patients preoperatively. Patients had no cavities but had a higher number of missing teeth in the post-operative phase of cancer

treatment. The oral hygiene of the patients improved during their cancer treatment.

Fig. 6. Incidence of *Candida* species in oral cancer patients at different stages of treatment, and in control subjects. In the patients at 8-12 weeks after the cancer treatment, 50% of the *Candida*-positive patients, species other than *C. albicans* was identified. wk: week; post-op.: post-operatively. Modified from Rautemaa *et al.* 2006 (155).



7.2 Novel filter paper sampling method (II)

The filter paper sample detected a higher number of CFU of aerobic and anaerobic bacteria compared to the swab. The mean of the total number of morphology types per sample recovered on FAA was 17.7 (SD±2.95) using the filter paper and 15.1 (SD±2.8) using the swab; these values equate to 0.13 (SD±0.02) and 0.10 (SD±0.02) colony morphology types of bacteria per square mm of oral mucosa, respectively. The difference was statistically significant on FAA ($P=0.0094$). On the BA, CLED and NV culture media the difference were not significant.

The filter paper sample did not significantly differ from the swab in the gram-positive/gram-negative ratio (median: filter 25.9; swab 62.3) or for the aerobic/anaerobic ratio (median: filter 2.3; swab 3.5). The mean of the total number of CFUs was 0.4×10^5 (SD± 0.5×10^5) per filter paper sample and 1.4×10^5

(SD \pm 1.7 \times 10⁵) per swab sample. The difference was statistically significant ($P=0.0001$). Both sampling methods did not differ in their sensitivity in detecting yeast colonization; both detected yeast from only one subject.

7.3 ACH PRODUCTION AND MICROBIAL COLONIZATION IN OLD AND OSCC (III)

The majority (68%) of the cultures from the patient samples produced mutagenic levels of acetaldehyde (>100 mM): 76% of all OSCC lesion samples; 72% of OSCC control samples; 61% of OLD lesion samples; 67% of OLD control samples; 60% of samples from control patients ($P = ns$). The mean level of acetaldehyde produced by all samples was 158 μ M (range 13-1000 μ M). The differences between patient groups and sampling sites were not statistically significant and there were no significant differences in the acetaldehyde production between clinically and histologically diagnosed oral lichen planus (OLP) and oral lichenoid reaction (OLR).

As determined by CFU per sample, in OSCC lesions were significantly higher numbers of microbes compared with the other patient groups ($P < 0.0001$; Figure 7). Likewise, the number of aerobic and anaerobic bacteria (CFU/sample) cultured from the OSCC lesion site was significantly higher compared with the other patient groups ($P < 0.0001$; Figure 7). There was no significant difference in the number of anaerobic bacteria per sample between lesion and control site of OSCC patients. In OLD patients, there was no significant difference in the microbial colonization density in lesion or control sites compared with control patients, or between OLD patients histologically diagnosed with OLP and OLR. Most bacteria cultured from samples from all patient subgroups were gram-positives. In OSCC patients, mean 4% in lesion site and 3% in control sites were gram-negatives. Likewise, in OLD patients, 12% in lesion sites and 8% in control sites were gram-negatives. In control patients 8% of the cultured bacteria were gram-negatives.

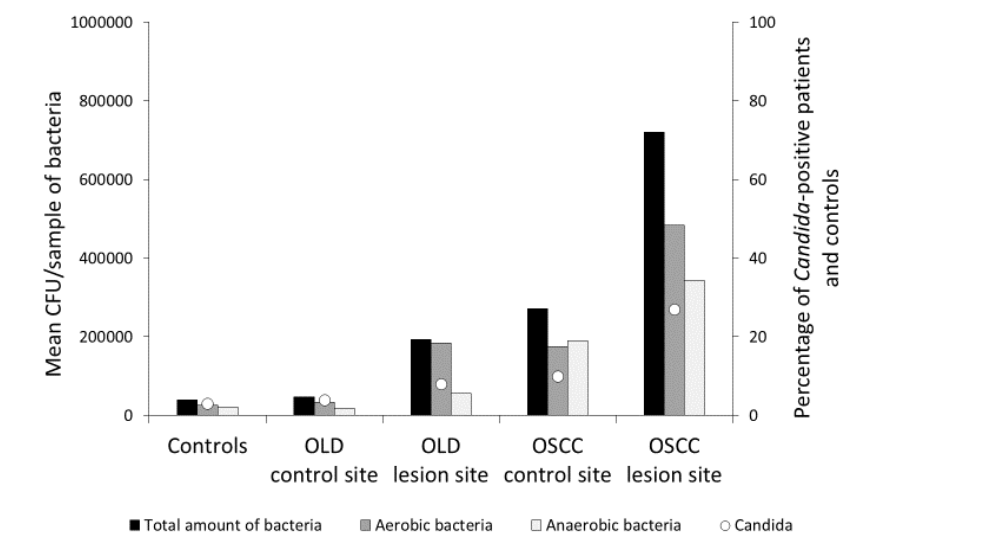
The density and frequency of *Candida* was higher in lesion sites compared to the control sites or samples from control patients. In OSCC patients, *Candida* was found from 27% of the lesion and 10% of the control sites and in OLD patients, *Candida* was found in 8% and 4% of the lesion and control sites, respectively. *Candida* was found in 3% of the control patients (Figure 7). Samples cultured from lesion sites of OSCC and OLD patients with *Candida* colonization produced significantly more frequently of mutagenic amount of acetaldehyde than cultures of patients with no candidal colonization ($P = 0.0008$). However, there was no

correlation between the total amount of cultivable microbes and acetaldehyde levels in any patient group or sample site.

The mean acetaldehyde production by microbes cultured from samples of smokers was significantly higher than from non-smoker samples ($P = 0.033$). In addition, microbes isolated from the lesion sites of smoking OSCC and OLD patients produced significantly higher amounts of acetaldehyde than microbes isolated from mucosal lesions of non-smokers (OSCC and OLD lesions combined) ($P = 0.0351$). However, non-smoker lesions had higher microbial density than smoker lesions (CFU/sample). In addition, on the lesion site (but not on control sites) of OSCC patients who did not consume any alcohol were significantly higher number of microbes compared to patients who consumed alcohol (mean 1,400,000 and 560,000, respectively; $P = 0.00063$).

No correlations between microbial colonisation or acetaldehyde production and TLR, NF- κ B and p53 immunohistochemical expression were found.

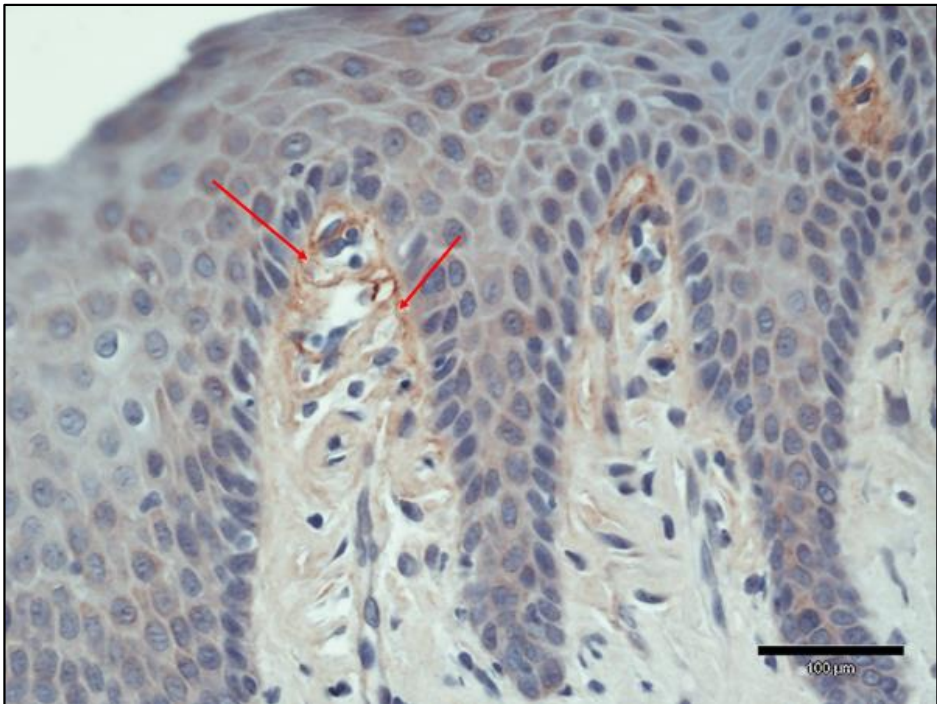
Fig. 7. Microbial colonisation (CFU/sample) in the different patient groups and sites presented as means. Significantly higher numbers of microbes were detected in lesions of OSCC patients compared to the control site and other patient groups ($P < 0.0001$). Likewise, the numbers of aerobic and anaerobic bacteria cultured from the OSCC lesion site were significantly higher compared to other sites ($P < 0.0001$). The proportion of *Candida*-positive samples of each sample type are shown as percentage. Modified from Marttila *et al.* 2013 (156).



7.4 TLR, NF- κ B AND P53 EXPRESSION IN OLD (IV)

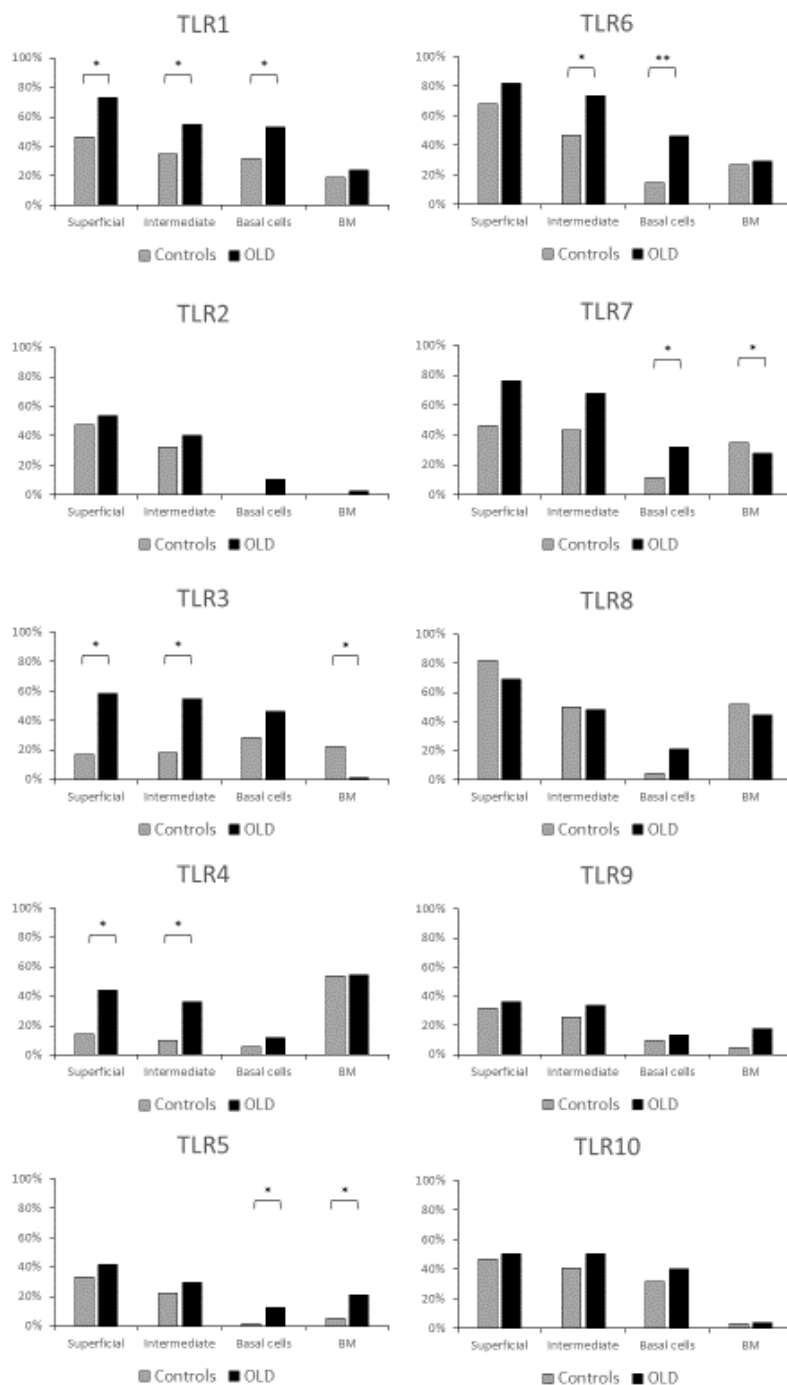
All TLRs, except TLR2, were expressed throughout oral epithelia in both OLD and control samples. TLR2 was not detected in the basal layer or the basement membrane (BM) zone of the control samples and was seen in only one OLD sample in the BM zone. Likewise, TLR3 expression was detected throughout the intermediate and superficial layers of the OLD and control samples but in only one of the OLD samples in the BM zone. In contrast, the staining intensity of TLR4 was significantly stronger in the BM zone compared to the other layers in OLD and control samples (Figure 8).

Fig. 8. Staining intensity of TLR4 in basement membrane zone in healthy control (red arrow, x40). The staining intensity of TLR4 was significantly stronger in the BM zone compared to the other layers in OLD and control samples. Modified from Rusanen *et al.* 2017 (1).



The expression of most of the TLRs had a trend of a gradual decrease from the superficial layers towards the basal layer. Expression was strongest in the superficial layer for all TLRs, except for TLR3 and TLR4 in the control samples, and TLR4 and TLR9 in the OLD samples. In general, the expression of several TLRs was markedly upregulated in the OLD samples compared to the control samples (Figure 9). In the superficial epithelium, the staining intensity for TLR1, TLR3 and TLR4 was significantly higher in OLD compared to the control samples ($P = 0.01$, $P = 0.002$, $P = 0.02$, respectively; Figure 9). Likewise, the staining intensity for TLR1, TLR3, TLR4 and TLR6 was significantly higher in the intermediate layer of OLD samples ($P = 0.03$, $P = 0.003$, $P = 0.03$, $P = 0.02$, respectively). Also, in the basal layer, the expression of TLR1, TLR5, TLR6, and TLR7 was increased in the OLD group when compared to the control group ($P = 0.02$, $P = 0.02$, $P = 0.0004$ and $P = 0.03$, respectively). In the BM zone, the expression of TLR5 was upregulated in OLD when compared to control samples ($P = 0.03$). In contrary, the expression of TLR3 and TLR7 in the BM zone was stronger in the control samples compared to the OLD samples ($P = 0.007$ and $P = 0.04$, respectively). All control samples showed a positive staining to all TLRs. The immunohistochemical expression of TLRs did not correlate with the age of the patients.

Fig. 9. Mean staining percentage of TLRs in epithelial layers and basement membrane (BM) zone. In general, the immunohistochemical expression of several TLRs was markedly upregulated in oral lichenoid disease (OLD). *: $P < 0.05$; **: $P < 0.001$. Modified from Rusanen *et al.* 2017 (1).



The expression of p53 increased from the superficial layers towards the basal epithelium in both control and OLD samples. Staining for p53 could not be detected in the superficial epithelial layers of the control samples, whereas two of the OLD cases showed weak staining (1–33% of cells) in this layer. In the control group, staining for p53 could be detected only in one sample in the intermediate layer and in five samples in the basal layer. The immunohistochemical expression for p53 was statistically stronger in basal layer compared to the intermediate layer in the control group ($P = 0.02$). In OLD samples, the staining intensity was significantly stronger in the intermediate layer compared to the superficial layer ($P = 0.002$) and in the basal layer compared to the superficial and intermediate layers ($P = 0.001$, $P = 0.003$, respectively). In general, the staining intensity of p53 was stronger in the OLD samples compared to the control samples in all layers. The difference was statistically significant in the basal and in the intermediate layers ($P = 0.002$ and $P = 0.009$, respectively).

The expression of NF- κ B decreased from the superficial epithelial layers towards the basal layer in both the control and OLD samples. Immunoreactivity for NF- κ B could be detected in all epithelial layers in both control and OLD samples. In the control samples, the staining for NF- κ B was significantly stronger in the superficial layer compared to the intermediate and the basal layers ($P = 0.001$, $P = 0.0005$, respectively). In the OLD samples, the expression of the NF- κ B in the superficial and in the intermediate layers was significantly stronger compared to the basal layer ($P = 0.002$, $P = 0.03$, respectively). In general, the staining for NF- κ B was more intense in the OLD samples compared to the control samples. The NF- κ B expression was significantly stronger in the OLD samples compared to the control samples in the intermediate layer ($P = 0.04$).

The staining intensity of p53 correlated positively with the staining intensity of NF- κ B and TLR1 in the basal layer of the OLD samples ($P = 0.02$ and $P = 0.02$, respectively). Accordingly, NF- κ B correlated positively with the immunoreactivity of TLR2 ($P = 0.05$). The correlations of p53, NF- κ B and TLRs in OLD are shown in figure 10. In the intermediate layer of the OLD samples, the staining intensity of p53 correlated positively with the immunoreactivity of NF- κ B ($P = 0.03$), TLR1 ($P = 0.009$) and TLR5 ($P = 0.03$). However, in the superficial layers of the OLD samples or in any layers of control samples, no significant correlations were seen in the staining intensities of TLR1–10, p53 and NF- κ B. There was no significant

difference in the staining intensity for TLR1-10, p53 nor NF- κ B in patients with OLP and patients with OLR or OLL in any parts of the epithelium.

Fig. 10. The statistically significant differences in the immunostaining of TLR1-10, p53 and NF- κ B in oral lichenoid disease (OLD) and control samples. *P*-values of less than 0.05 were considered statistically significant. Correlations between TLRs, p53 and NF- κ B were found only in OLD samples. BM zone: basement membrane zone.

Layer	OLD			Controls		Correlations	
	compared to controls			compared to OLD		in OLD samples	
	TLR	NF- κ B	p53	TLR		p53	NF- κ B
Superficial	TLR1						
	TLR3						
	TLR4						
Intermediate	TLR1	NF- κ B	p53			NF- κ B,	
	TLR3					TLR1,	
	TLR4					TLR5	
	TLR6						
Basal cell	TLR1		p53			NF- κ B,	TLR2
	TLR5					TLR1	
	TLR6						
	TLR7						
BM zone	TLR5			TLR3			
				TLR7			

8 DISCUSSION

This thesis showed that the frequency and density of *Candida* colonization was higher in OLD patients and OSCC patients undergoing cancer therapy compared to the healthy controls. Also, a shift from *C. albicans* to other *Candida* species in OSCC patients was seen, which is in accordance with previous studies (24, 157). These findings are likely to be a result of an altered host responses, cancer treatment and the use of fluconazole in OSCC patients. Although all *Candida* species cause a clinically similar mucositis, there are significant differences in the invasiveness and antifungal susceptibilities among species. The increase of the prevalence of non-*albicans Candida* species innately resistant to standard fluconazole treatment has significant antimicrobial stewardship implications, especially in high-risk patients. The use of non-azole treatment should be considered first line in order to avoid selection of these more difficult to treat species.

Most oral microbial samples produced mutagenic levels of acetaldehyde. Especially, samples cultured from OSCC and OLD patients with *Candida* colonization produced significantly more frequently of mutagenic amount of acetaldehyde than cultures of patients with no candidal colonization. This is in line with studies of other that have shown that *Candida albicans* can produce significant amounts of acetaldehyde (45, 158).

In our study a significantly higher number of aerobic and anaerobic bacteria were cultured from OSCC lesion sites compared to other patient groups. Also, in OLD lesion samples a higher proportion of gram-negative anaerobic bacteria were detected compared to other sample types. In addition, the *Candida* colonization was higher in lesions compared to the control sites and to samples from control patients. This in in accordance with the study of Nagy et al (51). A major factor for these findings would seem to be the irregularity of the lesion surface providing stagnant niches allowing oral commensals to become pathogenic when their balance is disrupted. However, acetaldehyde levels produced by the microbes cultured from the OLD or OSCC lesion site did not differ from the other subgroups. In addition, there was no correlation between acetaldehyde levels and the total amount of cultivable microbes in any patient group or sample site. Also, *Candida spp.* was not detected in all samples producing high amounts of acetaldehyde. This

indicates that the composition rather than the number of microbes is a significant factor that influences the production of carcinogenic level of acetaldehyde.

Our results supports the findings of other that the total amount of microbes isolated from smoking patients were higher compared to the non-smoking patients (42, 45, 159). Interestingly, the microbes isolated from the lesion site of smoking OSCC and OLD patients produced significantly higher amounts of acetaldehyde despite of a significantly lower microbial density compared to the lesion site of non-smoking patients. Poor oral hygiene and smoking influences immune response, and the healing capacity of the periodontium and oral mucosa leading to the changes of the microbial composition (160). It might be that among smoking patients the higher acetaldehyde production of microbes in the lesion site is rather due to the selection of more acetaldehyde producing microbes than the higher number of microbes on the lesion site. It has been shown that acetaldehyde is toxic when added to at high concentrations to cell cultures (161, 162). Thus, the selection of acetaldehyde producing microbes may be due to the constant exposure of tobacco smoke that contains acetaldehyde which in turn favours microbes that are more tolerant to the acetaldehyde. Also, in the presence of alcohol, the acetaldehyde production is increased that might amplify the selection of acetaldehyde producing microbes.

Recent studies of the increased acetaldehyde amount among smokers and alcohol consumers are based mainly on uncultured saliva or blood samples (36, 42, 43, 45). This approach does not give site specific results of the lesion specific microbes and its acetaldehyde production. Also, oral swabs for culture is a commonly used sampling method but it gives semiquantitative results at the best. In our study the measurement protocol for microbial acetaldehyde production was based on standardized sample size rather than the number of microbes. Our findings underline the importance of using an appropriate lesion specific and quantitative sampling method, in addition to performing accurate microbiological diagnostics, in these patients.

In this thesis, the staining intensity of several TLRs was markedly stronger throughout the epithelium of OLD compared to the control samples. In addition, both OLD and control samples had a trend of a gradual decrease in most of the TLRs from the superficial layers towards the basal layer. In epithelium, the staining intensity of TLR1, TLR3, TLR4, TLR5, TLR6 and TLR7 were significantly higher in OLD compared to healthy controls. Literature on the participation of

TLRs in the pathogenesis of OLD is relatively scarce. However, significant increase of TLR1, TLR2, TLR4, TLR7, TLR8 and TLR9 has also previously been found in OLP compared to healthy controls (114, 117-119, 163). The differences between these findings may be due to the differences in the antibodies and the methodology used. In addition, different scoring has been used. In this thesis the basement membrane zone was recorded separately.

Lipopolysaccharides (LPS) of gram-negative bacteria and mannan of *Candida* are recognized by TLR4. Thus, the higher immunostaining of TLR4 in superficial and intermediate layers of OLD samples might be related to our microbiological findings where OLD samples showed a higher *Candida* colonisation and a higher proportion of Gram-negative anaerobic bacteria compared to other sample types. When comparing the different layers in OLD and in healthy controls, our results revealed a strongest immunostaining of TLR4 in the basement membrane zone compared to the epithelium. In addition, the immunostaining of TLR4 in the basement membrane zone was stronger in healthy controls compared to OLD even though, the difference was not statistically significant. According to the study of Zhang *et al.* (126) the TLR4-stimulated oral keratinocytes inhibited the proliferation of OLP CD4+ T-cells and OLP CD8+ T-cells, and simultaneously prompted their apoptosis. It might be that TLR4 especially in the basement membrane zone play role in the pathogenesis of OLD.

In OSCC patients, according to our yet unpublished data, the immunostaining of TLR4 was significantly lower compared to the same of the healthy controls in the basement membrane zone. However, in the epithelium were no significant differences in the staining intensity of any TLRs between OSCC and healthy controls. In OSCC patients the immunostaining of TLR1-10 were analysed from the healthy appearing mucosa near the cancer. Interestingly, according to our yet unpublished data, there was a statistically significant association between TLR4 staining intensity in the basement membrane zone and *Candida* colonisation in OSCC patients. In addition, there was an association between TLR4 immunostaining in basal layers and acetaldehyde production in OSCC patients. According to the meta-analysis of Hao *et al.* 2018 the elevated expression of TLR4 in cancer patients is associated with poor overall survival and shorter disease-free survival (68). In addition, high TLR4 expression and poor survival rate in OSCC patients has been reported (164). On the other hand, in several cancers (hepatocellular carcinoma, breast cancer, colorectal cancer and lung cancer) the

downregulation of TLR4 has reported to inhibit the tumour growth and suppresses the metastasis of carcinoma (165-168). Our results indicate that the elevated TLR4 immunostaining in OSCC patients is due to the increased number of acetaldehyde producing microbes including *Candida* spp., which have been shown to be heavy acetaldehyde producers (169). It is still uncertain and debatable whether microbial invasion is a causal or secondary event in oral premalignant and malignant lesions (48, 51). However, our results indicate that acetaldehyde and *Candida* colonisation have an impact on TLR4 expression that may play a role in OSCC pathogenesis.

In this thesis, the staining for NF- κ B was more intense in the OLD samples compared to the control samples, especially in the intermediate layer. This is in line with the study of Ge *et al.* (163). Likewise, consistent to previous studies, the staining intensity of p53 in the intermediate and basal cell layers was significantly stronger in OLD samples compared to the healthy controls (170, 171). According to our results, there was a positive correlation between p53, TLR5 and NF- κ B in the intermediate layer of OLD samples even though, the immunostaining of TLR5 in the epithelium did not show significant differences between OLD and healthy controls. However, the staining intensity of TLR5 was significantly higher in the basement membrane zone of the OLD samples. This may be due to a polymorphism and a change in the function of TLR5 that induce the expression of p53. The data of the study of Rutkowski *et al.* (172) indicated that TLR5 signalling is sufficient to drive systemic tumour-promoting inflammation due to the polymorphism of TLR5. The only known natural ligand for TLR5, flagellin, is exclusively of bacterial origin. However, we did not find any correlations between the microbial samples and the immunostaining of TLRs.

In addition to the hitherto known infection sources, plaque, caries and chronic inflammatory periodontal disease lesions, our findings supports the studies of others that the OSCC and OLD lesion itself may increase the local and systemic infection risk and may increase risk of the malignant transformation of OLD lesions (62, 109, 135). Oral lichen planus is a systemic disease and even the healthy appearing mucosa is diseased, and the malignant transformation is not restricted to the site of the lesion (104). It would be of interest to conduct a longitudinal study using a site-specific and quantitative sampling method to investigate the changes in the microbial flora and its acetaldehyde production on

OLD lesions compared to the healthy appearing mucosa whether a connection could be found possibly leading to the malignant transformation of OLD.

8.1 METHODOLOGICAL CONSIDERATIONS

Microbiological analyses

In this thesis, the microbes were analyzed using culture-based techniques and microbial colonies were classified as Gram-positive or -negative and as aerobic or strictly anaerobic. It is well known that culture-based methods have a number of limitations and may lack sensitivity compared to molecular methods. This is particularly an issue regarding fastidious anaerobic bacteria (173). It has been estimated that up to half of oral bacteria are not culturable using standard techniques and that gram-negative species are particularly underestimated compared to the estimates obtained by qPCR (5, 174). The identification of bacterial and yeast isolates by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry would also have been more accurate. However, the identification by MALDI-TOF mass spectrometry is usually performed on microbial biomass extracted from a non-selective culture medium which is also prone to biases. In addition, the MALDI-TOF mass spectrometry has been in a regular use only recently. However, when delays from sampling to plating are minimized like in this study setting, microbial culture is otherwise a robust method and a useful diagnostic tool for the detection and identification of viable organisms, including many known pathogenic species (173).

The limitation to the study I is a relatively small number of samples and the lack of statistical analyses. However, the results of our study showed that the site of sampling for oral yeast cultivation is crucial for correct diagnosis in patients in whom surgical operations may have deformed the anatomy of the oral cavity. Dental plaque on tooth surface was found to have the highest density of *Candida* colonization of all sites sampled. Thus, it was found to be the most significant source of *Candida*, which emphasizes the role of dental care protocols in these patients. However, there is a risk of overdiagnosis and treatment when sampling dental plaque from tooth surfaces, as the amount of yeast cultured is used for treatment decisions in these patients. Sampling dental surfaces may lead also to false negative results if the sampling has performed after a dental care protocol, as we noted in our study. In addition, due to the cancer treatment the patient might be edentulous. The post-operative antimicrobial treatment should be targeted

against pathogens which should be identified using a reproducible sampling method.

In the past decade comprehensive understanding of the oral microbial diversity has resulted from improvement in the techniques used in identification of oral microbes. Despite of the improvement of these techniques only little attention has been given to the first steps in diagnostics i.e. sampling methods. Comparison of the findings of various clinical studies is difficult due to the different methodologies used. An ideal sampling method that would give representative microbial results from site of the active disease process should be minimally invasive, site specific, repeatable and quantitative. A sterile swab is the most commonly used method for sampling a mucosal lesion. However, it is influenced by a number of variables, such as the area sampled, the force applied to the mucosa, and retention of the sample in the swab matrix during plating. In studies II and III, these variables are controlled by using a filter paper for sampling. The type of filter paper was selected mainly based on its pore size allowing capillary flow of saliva into the hydrophilic filter paper creating a gentle suction and thereby releasing adherent microorganisms without rubbing but not sucking the microbes into the the matrix of the filter paper. In addition, the sample area of the filter paper is constant which allows quantitative reporting. Although the type of the filter paper was selected not to interfere with PCR, this was not tested in the current study and remains to be tested and validated in future work.

There are some limitations to the study III. The sample size in different sub groups in respect of drinking and smoking habits was too small for statistical comparison. Moreover, the ACH analyses were done using microbial growth on FAA plates which had been plated using filter papers after they had been washed in a sterile saline used for the microbial analysis. It is likely that some microbes became washed off more than others potentially affecting the ACH analysis. However, all samples were analysed in a similar manner and we found clear differences between different patient groups and between lesion and healthy appearing mucosa of the same patient.

Immunohistochemical staining

We acknowledge some limitations in this thesis, such as the difference in age between the OLD patients and healthy controls. This could partly be attributed to the fact that OLD is more common in the adult population, whereas the control

samples taken during the surgical removal of third molars is mainly performed in young adults. However, it is unlikely that the age gap would have a major impact on the findings because, in contrast to adaptive immune responses, the innate responses are not generally significantly affected by aging (175). In fact, in this thesis, we did not find any age-related correlations with the immunostaining of any TLRs.

The main limitation of the study IV is that only immunohistochemical (IHC) staining was used to compare the level of expression of TLRs, NF- κ B and p53 between the OLD and healthy control samples due to limited amount of tissue available. IHC staining is an excellent detection technique for visualizing the distribution and localization of biomarkers and differentially expressed antigens in different parts of tissue levels. IHC staining also gives an indication of the amount of the given antigen and it preserves the histologic architecture which enables the pathologist to confirm that the positive cells are the cells in question. However, its major disadvantage is that it cannot determine if the observed biomarkers are active and functional in the tissue. Moreover, by IHC staining, it cannot be distinguished whether the p53 is a wild-type or in an inactive form. To determine the aetiology of OLD, additional techniques, such as measuring mRNA levels by qPCR, should be used to investigate the factors leading to T-cell accumulation and the changes in chemokine, chemokine receptor and adhesion molecule expression. However, qPCR does not provide site specific information.

The validity of immunohistochemistry in diagnostic pathology depends on the quality of the immunostains and there is a high degree of variability in the way tissues are initially prepared. These variations include tissue fixation and processing, unmasking of epitopes, and sensitivity of the detection system (176). Although we do not have exact information about how the specimen were handled, all the biopsies were taken in the hospital or in the university clinic and the fixation and processing were carried out by a fixed routine. The optimal primary antibody concentrations for immunohistochemistry was selected based on our pilot experiments.

9 SUMMARY AND CONCLUSIONS

The results of this thesis have led to the following conclusions:

- I. In oral cancer patients the optimal sampling method and site need careful attention, in whom surgical operations may have deformed the anatomy of the oral cavity. In this limited patient population, the optimal sampling site for *Candida* was found to be the labial sulcus. We found that *Candida* colonisation was higher in OSCC patients compared to the controls even before the cancer treatment. After cancer treatment, the incidence was found to be increased and a shift from *C. albicans* into other *Candida* species was found.
- II. The novel filter paper sampling method was found to be an ideal technique for obtaining quantitative data from defined areas of the oral mucosa. The method is easy to perform in a routine setting.
- III. Based on results of filter paper sampling method we found that the bacterial composition on OSCC and OLD lesions differed from control site and healthy controls. Most of the cultures from the patient samples produced mutagenic levels of acetaldehyde. The *Candida* colonization was higher in OSCC and OLD lesions and patients with *Candida* colonization produced significantly more frequently of mutagenic amount of acetaldehyde. However, there was no correlation between acetaldehyde levels and the total amount of cultivable microbes. Our results indicate that the composition rather than the number of microbes is a significant factor that influences the production of carcinogenic level of acetaldehyde. This underlines the importance of using a site-specific sampling method that gives quantitative results for microbial analysis.
- IV. We were able to map all TLRs, p53 and NF- κ B, and their co-localization in the epithelium and BM-zone. The increased immunostaining of several TLRs in OLD compared to healthy controls indicate an important role in this mucocutaneous oral disease. To the best of our knowledge, this thesis is the first to study all TLRs in the basement membrane zone. According to our findings, it is likely that staining of several TLRs in the basement membrane zone is due to the presence of soluble TLRs fragments produced by the basal cells. The role of soluble TLR forms in the basement membrane zone calls for further studies.

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